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(54) Title: NOVEL ESTROGEN RECEPTOR β AND ISOFORMS

(57) Abstract

The present invention provides the amino acid and nucleotide sequence of the complete estrogen receptor beta ($ER\beta_c$) gene and related protein sequences. Based on this disclosure, the present invention provides methods for identifying agents that block or augment $ER\beta_c$ mediated transcriptional regulation, methods to determine whether $ER\beta_c$ or its isoforms is being expressed in tissues or cells and methods of identifying and using agents that block the transcriptional regulation of genes by $ER\beta_c$ or its isoforms, which in turn modulates other biological and pathological processes.

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NOVEL ESTROGEN RECEPTOR β AND ISOFORMS

FIELD OF THE INVENTION

The present invention relates to a novel complete mammalian estrogen receptor β, referred to as $ER\beta_c$, its polypeptide sequence, the nucleic acid sequence encoding $ER\beta_c$ and methods of making or expressing $ER\beta_c$. The present invention also relates to methods of screening for drugs which modulate the interaction of estrogens and $ER\beta_c$ as well as methods of diagnosing and/or treating diseases involving $ER\beta_c$ or its isoforms. This application is related to U.S. Provisional applications 60/053,869 and 60/054,210, which are herein incorporated by reference.

BACKGROUND OF THE INVENTION

A. Estrogen

Estrogens are a class of naturally occurring steroid hormones which are produced in the ovaries and other tissues of the body including the testis. Estrogens are known to directly influence the growth, differentiation and function of specific target tissues and organs in humans and animals. These specific tissues and organs also include the mammary gland, uterus, prostate, pituitary, brain and liver. Estrogens also play an important role in bone maintenance and in the cardiovascular system, where estrogens have certain cardio-protective effects. In bone, both osteoclasts and osteoblasts have been reported to respond to estrogens with estrogen withdrawal leading to increased turnover and bone loss. A variety of naturally occurring and chemically synthesized estrogens have been identified and characterized, perhaps the best known of which is the endogenous estrogen, estradiol-17 beta (also known as E_2).

B. Estrogen Receptors

Estrogens, as a class of hormones, act by binding to the ligand binding domain (LBD) of an intracellular protein identified as an "estrogen receptor" (ER). The presence of this intracellular ER provides and accounts for both cell proliferation and protein synthesis by estrogen-dependent cells. In the absence of the estrogen hormone,

the estrogen receptor is biologically inactive both *in vivo* and *in vitro*; and, if the cells or tissues are homogenized and fractionated into cytosol and nuclear fractions, the estrogen receptor is found in the nucleus and may also be detected the cytosol.

The known estrogen receptors are members of the well studied family of gene regulatory proteins referred to as the steroid hormone receptor family. Nuclear receptors, such as steroid hormone receptors, have a modular structure with six distinct regions. The N-terminal domain is the A/B region which includes a non-ligand dependent activation function (See Fig. 1a). The C region is the DNA binding domain (DBD). The D region contains nuclear localization signals. Finally, the E domain contains the ligand binding domain (LBD) and the ligand-dependent transaction function. Kuiper *et al.*, Endocrinology 138(3): 863 (1997); Tremblay *et al.*, Mol. Endocrin 11(3): 333 (1997). The central DBD is typically about 100 amino acids. Like the other members of the steroid-hormone receptor family, estrogen receptors are activated by the binding of estrogen to the C-terminal LBD. The receptor proteins enable cells to respond to various lipid-soluble hormones by activating or repressing specific genes, through the interaction between the steroid hormone and its receptor. Steroid hormone receptors are distinguishable from other nuclear receptors in a number of respects, including the nature of their ligands, their association (in the unliganded state) with a repertoire of heat-shock proteins and the fact that they may bind to hormone response elements as homodimers. Mosselman *et al.*, FEBS Letters 392: 49 (1996); Komm *et al.*, Science 241: 81 (1988); Burch *et al.*, Mol. Cell. Biol. 8(3): 1123 (1988).

The conventional model for steroid hormone action has assumed that steroid hormone receptors act as transcriptional regulators only when complexed with their ligands. It has, however, become evident that the majority of steroid receptors are present in the cell nucleus even in the absence of ligand. The presence of the receptors in the nucleus, despite the absence of hormone, suggests possible additional regulatory functions for the receptor in its unbound state. For example, the thyroid hormone receptors (TR) have a dual regulatory role: in the presence of hormone they function as

transcriptional activators, whereas in the absence of hormone, TRs are response element (TRE) specific transcriptional repressors.

The first estrogen receptor discovered was ERα, which was known for the past ten years merely as ER. The human ER (hER) is composed of 595 amino acids in its unbound state and is approximately 67,000 Daltons. In the absence of estrogen-binding, the ERα protein can be located *in vitro* within the cytosol.

Transcription of ERα occurs from two separate promoters, P0 and P1, although no functional mapping has been previously published. P1 represents the major ERα transcriptional start site. The P1 start site is predominantly utilized in human mammary epithelial cells (HMEC) and is the major start site in ERα-positive human breast carcinomas. Multiple start sites have been identified for the P0 promoter. Studies of the murine ERα gene identified 10 start sites spanning approximately 60 bases, and there is a start site at - 1,994 (from the P1 start site) in human cells, which would agree closely with the major murine P0 start site. Transcription from the P0 promoter is characteristic of human endometrial tissue and can account for 12 to 33% of ERα transcription in breast carcinoma cells. The genetic regulatory control elements of the recently discovered *ERβ* gene have yet to be delineated. Kuiper *et al.*, (1996 and 1997); Tremblay *et al.*, (1997); and Mosselman *et al.*, (1996). It remains to be determined whether the *ERβ* gene contains regulatory elements, such as promoters and enhancers, that are similar or function in a manner analogous to those described for ERα.

In soluble systems and under set conditions, the ERα protein can be found in various molecular forms with sedimentation coefficients of 8S, 5S or 4S as determined by sucrose
density gradient analysis. The 8S form of ERα protein is believed to be the inactivated, untransformed form of ERα protein associated with the unbound, inactive state of estrogen receptor in the absence of estrogen. The 4S ERα protein is a monomeric protein molecule that can be generated from the 8S form *in vitro*. The 4S form binds to both nuclei and DNA-cellulose *in vitro*; it is generally termed the "activated but untransformed" estrogen receptor protein. The 5S form of ERα is a dimeric protein

molecule, which is created by the conversion of the 4S ERα protein via a bimolecular reaction. It is generally believed that the 5S form of ERα protein is both "activated and transformed," and therefore is the biologically active entity which binds to the DNA within the nuclei. Moreover, it is also this 5S form which is found associated with the nuclei subsequent to the administration of estradiol *in vivo*. Already it has been demonstrated that both ERα and ERβ can form heterodimers (Kuiper and Gustafsson, FEBS 410: 87 (1997)).

Although the precise interactions between ERα and estrogens remain poorly understood, the generally accepted mechanism of action and sequence of events is believed to be as follows: When an estrogen, such as estradiol 17 beta (E_2), is introduced to the target cells and tissues, there is specific binding between the estrogen and the ERα protein which results in the formation of an estrogen/ERα protein complex. Also, at a time subsequent to hormone binding, a process termed activation and/or transformation ensues leading to the formation of functional estrogen/hormone receptor complexes possessing a high affinity for the nuclear components, the DNA of the target cell. Once the hormone/receptor protein complex is physically formed, it binds to the chromatin at specific binding sites on the chromosomes and regulates messenger ribonucleic acid (mRNA) transcription. If transcription is up-regulated, new messenger RNA (mRNA) is synthesized, chemically modified and exported from the nucleus into the cytoplasm of the cell where ribosomes then translate the mRNA into new proteins; the hormone/receptor protein complex can also down-regulate mRNA transcription. This constitutes the well recognized estrogenic effect that occurs within cells and involves the regulation of new protein synthesis and concomitant new cell growth/proliferation or differentiation. It remains unclear whether ERβ shares the same mechanisms of action and in the same order as have been demonstrated for ERα. Certainly, the localization of ERβ along with the manner in which it modulates transcription will be at least grossly similar to ERα; however, affinities for certain DNA sequences, as well as receptor ligands likely will differ between ERα and ERβ, as there

is a 97% and 60% identity respectively between the DBD and LBD sequences between the two estrogen receptors. Tremblay *et al.*, (1997).

The clinical significance of the ER in the management of breast cancer is well known. Tamoxifen, a substituted triphenylethylene antiestrogen, is a partial antagonist that is used in the management of ER-positive breast tumors. Gallo *et al.*, Semin. Oncol. 24: S1 (1997). Generally, the expression of the receptor is usually associated with a better prognosis and is less metastatic. Bonetti *et al.*, Breast Cancer Res. Treat. 38(3): 289 (1996). However, in many cases the tumors are either ER-negative or contain splice variants that are commonly biologically inactive. Hence, there is interest to understand how ER gene regulation, as well as the editing of the ER message, contribute to the development of mammary cancer and its clinical outcome with chemotherapy drugs such as tamoxifen. For more background, see Gallo *et al.*, (1997); Kangas, Acta Oncol. 31(2): 143 (1992); Evans *et al.*, Bone 17(4S): 1815 (1995); Safarians *et al.*, Cancer Res. 56(15): 3560 (1996).

More recently, estrogen receptors have been linked to bone loss associated with postmenopausal osteoporosis. Paralleling that discovery has been the fact that certain antiestrogens (*e.g.*, tamoxifen, raloxifene, droloxifene and tamoxifen methiodide), which by definition block the actions of estrogens, stimulate only the skeletal muscle tissues and have no corresponding stimulatory effect in the uterus or mesometrial fat. Somjen *et al.*, J. Steroid Biochem. Mol. Biol. 59: 389 (1996); Grasser *et al.*, J. Cell Biochem. 65: 159 (1997). These antiestrogens have been termed selective estrogen receptor modulators (SERMs); they typically possess estrogen agonist-like activity on bone tissues and serum lipids, while displaying potent estrogen antagonist properties in the breast and uterus. The observed paradoxical effects observed between the different estrogen receptor agonists and antagonists most likely corresponds to response differences to the antiestrogens between ERα and ERβ. Tremblay *et al.*, 361 (1997).

Estrogen receptors are also present in human and rat prostate, as evidenced by ligand binding studies. In contrast to androgen receptors, the major part of the estrogen receptors are localized in the stroma of the rat prostate, although the epithelial cells of

the secreting alveoli contain ER. Estrogens are, in addition to androgens, implicated in the growth of the prostate, and consequently estrogens have been implicated in the pathogenesis of benign prostatic hyperplasia. Habenich *et al.*, J. Steroid Biochem. Mol Biol. 44: 557 (1993); Kuiper *et al.*, PNAS 93: 5930 (1996). Diethylstilbesterol (DES), a stilbene estrogen with an increased affinity for ER, is used to treat prostatic hyperplasia and carcinoma. Goethuys *et al.*, Am. J. Clin. Oncol. 20(1): 40 (1997); Aprikian *et al.*, Cancer 71(12): 3952 (1993). Therefore, identifying the tissues and diseases that express ERβ likely will prove helpful in the treatment of diseases involving ERβ.

Estrogen has also been demonstrated to prevent osteoporosis. Postmenopausal osteoporosis, the most common bone disease in the developed world, is associated with estrogen deficiency. This deficiency increases generation and activity of osteoclasts, large multi-nuclear cells involved with bone resorption. Estrogen has been demonstrated to down-regulate osteoclast formation and function. Tamoxifen has been demonstrated to possess estrogenic effects on bone resorption likely through tamoxifen-induced osteoclast apoptosis. Hughes *et al.*, Nat. Med. 2(10): 1132 (1996). Isolation of additional reagents that inhibit progression of osteoporosis would be beneficial in treating postmenopausal women suffering from the disease.

C. ERβ

Following the cloning of estrogen receptor α (ERα) 10 years ago, there was general acceptance that only one ER gene existed and consequently only one subtype of ER, ERα. This contrasted sharply with other members of the nuclear receptor superfamily, where multiple forms have been reported, *e.g.*, thyroid hormone receptor (TR) α and β and retinoic acid receptor (RAR) α, β, and γ.

Recently, a novel rat ER cDNA was cloned from rat prostate and ovary tissues and named ERβ subtype to distinguish it from the previously cloned ER cDNA, now named the ERα subtype. ERβ was partially isolated from cDNA libraries from human testis, mouse ovaries and rat prostate, which are not generally considered to be major estrogen target tissues. The estrogen receptor subtype initially discovered was termed

ERβ, but for purposes of this invention will be termed the incomplete ERβ (ERβ$_i$) to differentiate it from the complete ERβ (ERβ$_c$ or ERβ-3) of the present invention, or the three claimed alternatively spliced isoforms (ERβ-1, ERβ-2 and ERβ-4) of this invention. Mosselman *et al.*, (1996); Kuiper *et al.,* (1996 and 1997); Tremblay *et al.*, (1997). "ERβ-3" refers to the sequence as isolated from mouse ovaries or its analogous sequence in other mammalian species. "ERβ$_c$" refers to the sequence that encodes the complete ERβ, which includes the novel 192 bp at the 5' terminus of exon 1 and the newly described exon 5B; ERβ$_c$ includes ERβ-3, the complete sequence that encodes the nine exons of murine ERβ. ERβ$_i$, as characterized using the clones obtained from mouse ovary tissue, encodes a protein that has a molecular weight of approximately 62 kDa and has a 60 kilobase (Kb) gene size. The isolated mouse ERβ$_i$ gene, called *Estrb* by Tremblay *et al.*, mapped to the central region of chromosome 12; the central region of mouse chromosome 12 shares homology with human chromosome 14q, suggesting that *Estrb* may lie here as well. Tremblay *et al.*, (1997).

The ERβ$_i$ cDNA encoded a predicted protein of 485 amino acids and had a calculated molecular weight of 54,200. Kuiper *et al.*, (1997). This protein, described by Kuiper *et al.*, as Clone 29 (herein ERβ$_i$), displays high affinity binding of estrogens, and in a transactivation assay system, it activates expression of an estrogen response element (ERE) containing a reporter gene construct in the presence of estrogens. Kuiper *et al.*, (1996). Alignment of the ligand binding domain (LBD) of ERα (rat, mouse and human) and ERβ$_i$ (rat) uncovered various regions of conservation, whereas other segments are non-conserved. Kuiper *et al.*, (1996). The DNA binding domain (DBD) and C-terminal LBD of ERβ$_i$ is highly homologous to the rat ERα. Kuiper *et al.*, (1997); Tremblay *et al.,* (1997).

ERβ$_i$ was isolated in an effort to clone and characterize novel nuclear receptors or unknown isoforms of existing receptors. Degenerate primers were designed based on conserved regions within the DBD and LBD of nuclear receptors. Using these primers in conjunction with Polymerase Chain Reaction (PCR), rat prostate mRNA was amplified. One targeted tissue was the prostate, an organ of interest given the high

incidence of prostate cancer and benign prostatic hyperplasia. Nearly all prostate tumors eventually become androgen-independent, at which point they are beyond clinical control. Kuiper *et al.*, (1996). Therefore, the possibility existed that, apart from androgens, other (steroid) hormones or locally produced factors interacted with nuclear receptors and modulated the cell proliferation, differentiation and apoptosis of the normal prostate. For example, human testicular receptors 2 and 4 (TR2 and TR4) and the estrogen receptor (ER)-related receptors (ERR1 and ERR2) are examples of orphan nuclear genes whose receptors are expressed in the prostate. Kuiper *et al.*, (1996).

Tissue expression of $ER\beta_i$ revealed additional differences from $ER\alpha$ expression. Some tissues contain exclusively $ER\alpha$ (*i.e.*, uterus, pituitary, epididymis, and kidney). Other tissues display equal or greater levels of $ER\beta_i$ RNA and may be expressed preferentially in the different cell types of an organ (*i.e.,* ovary and prostate). Kuiper *et al.*, (1996). In brain, $ER\beta_i$ appears to be a conspicuous fraction of the ER subtype RNA. Although Northern blots did not detect $ER\beta_i$ expression in peripheral blood lymphocytes, the initial PCR fragment of $ER\beta_i$ cloned by Mosselman was acquired from these cells. Thus, the $ER\beta_i$ subtype may play a significant role in estrogen action in brain, ovary, prostate, hypothalamus and possibly other tissues. Mosselman *et al.*, at 52 (1996); Byers *et al.*, Mol. Endocrinol. 11(2): 172 (1997); and Shughrue *et al.*, Steroids 61(12): 678 (1996).

In addition to differences in tissue expression, the order of competition for physiological estrogens and stilbene estrogens, which form a diphenolic resonance structure, for $ER\alpha$ versus the $ER\beta_i$ isoform was also observed to vary. Kuiper *et al.*, (1997). These differences may result from the protein sequences differences observed between $ER\alpha$ and $ER\beta_c$, as demonstrated in the comparison of the two murine subtypes in Figure 6. However, the order of affinity for the tested triphenylethylene antiestrogens however, was the same for both subtypes: 4-OH-tamoxifen >> nafoxidine > clomifene > tamoxifen. Kuiper *et al.*, (1997). Such ligand binding differences most likely, portend different drug therapies for $ER\alpha$ versus $ER\beta$ dependent disease. Another incongruity between the two ER subtypes is the agonistic-antagonistic difference in response to estrogens. This observed paradoxical disparity may relate not only to binding affinity,

but, more importantly to the presence of specific activation function domains located in $ER\beta_c$ (*e.g.*, AF-1 or AF-2). Tremblay *et al.*, (1997).

Substantial interest exists in determining the individual and perhaps combined roles that both ERβ and ERα play in carcinomas, increased estrogen turnover and bone loss. For example, tamoxifen augments bone growth, whereas it is an antagonist to ERα positive breast cancer. Gallo *et al.*, (1997); Delmas *et al.*, J. Clin. Oncol. 15: 955 (1997). This incongruous observation with tamoxifen administration may arise from the drug's different interactions with ERα and ERβ. The mixed agonist-antagonist or pure antagonist actions observed with antiestrogens may result more specifically from binding differences between the activation function domains, AF-1 and AF-2. For example, studies using the estrogen antagonists 4-hydroxytamoxifen (OHT) and ICI 164,384 indicated that although both compounds blocked estrogen effects, their mode of action differed: the mixed agonist/antagonist OHT inhibited only AF-2 function, while the pure antiestrogen ICI 164,384 inhibited activation by both AF-1 and AF-2. Using the mouse $ER\beta_i$, all antagonists tested effectively inhibited E_2 activity. In contrast to ERα, OHT displayed no agonistic activity on $ER\beta_i$. Tremblay *et al.*, (1997). Therefore, once the underlying ER subtype responsible for a particular disease state is determined (*e.g.*, ERα positive breast cancer), one may have a more accurate means of prognosticating the estrogen receptor related disease outcome; one may accurately follow therapies; one may develop gene specific and isoform specific therapies targeting diseases influenced by ERα and/or ERβ; and one may provide for opportunities for varying the aggressiveness of the therapy.

SUMMARY OF THE INVENTION

The present invention is based, in part, on the isolation and identification of the complete murine (m) estrogen receptor β gene (*mERβ-3*) and two alternatively spliced isoforms, *e.g.*, *mERβ-1* and *mERβ-2* and a third isoform isolated from rat (r) ovaries, *rERβ-4*. More broadly, the invention relates to the corresponding $ER\beta_c$ gene (including the human gene) and to certain mammalian receptors (denoted herein as ERβ-1, ERβ-2,

ERβ-3 and ERβ-4). The $ER\beta_i$ sequence has been published by other laboratories, which had prematurely claimed that $ER\beta_i$ represented the complete *ERβ* gene ($ER\beta_c$).

The present invention further provides nucleic acid molecules that encode the mERβ-1, mERβ-2, mERβ-3 and mERβ-4 proteins. Such nucleic acid molecules can be in an isolated form or can be operably linked to expression control elements or vector sequences.

The present invention also provides methods of identifying other alternatively spliced forms of the *mERβ-3*, the analogous *mERβ-3* and corresponding $ER\beta_c$ as expressed in different animal species or additional ER subtypes. Specifically, the nucleic acid sequence of *mERβ-3* can be used as a probe or to generate PCR primers to identify nucleic acid molecules that encode other members of the $ER\beta_c$ family of proteins. The nucleic acid molecules encoding *mERβ-1, mERβ-2, mERβ-3* or *rERβ-4* can be used to identify and isolate the *ERβ-3* gene or corresponding $ER\beta_c$ in other mammalian species, and has been used to isolate the *ERβ-3* analog in human DNA.

The present invention further provides antibodies that recognize and bind to the $ER\beta_c$ protein or the mERβ-3 protein or its isoforms. Such antibodies can be either polyclonal or monoclonal. Particularly preferred are antibodies that are specific for the complete receptor protein, $ER\beta_c$, as opposed to antibodies against the previously known receptors, *e.g.*, ERα and $ER\beta_i$. More specifically, the invention claims an anti-peptide antibody that distinguishes between $ER\beta_i$ and $ER\beta_c$. Antibodies that bind to the $ER\beta_c$ protein can be utilized in a variety of diagnostic and prognostic formats and therapeutic methods. Alternatively, antibodies that can distinguish between the complete form, $ER\beta_c$, and its isoforms may also be useful for purposes of diagnosis and treatment of ERβ subtype based disease.

The present invention further provides methods for reducing, blocking or augmenting the association of an estrogen and other agonists and antagonists with the $ER\beta_c$ protein. For example, the association of an ERβ-3 protein with a cytoplasmic signaling partner, such as estradiol, can be blocked or reduced by contacting the ERβ-3 protein with a compound that blocks the binding of estradiol or other estrogen-like

agonists or antagonists (*e.g.*, estrogens, stilbene estrogens or triphenylethylene antiestrogens). Tora *et al.*, Cell 59: 447 (1989); Berry *et al.*, EMBO 9: 2811 (1990). Additionally, as the proteins are allosteric, the association of the ligand with ERβ can also be influenced, in theory, by the dimer partner. Therefore, identifying agents that modulate ERβ dimerization may pose another means of manipulating ERβ regulation.

Blocking the interaction between the ligand and ERβ-3 or one of its isoforms can be used to modulate biological and pathological processes that require such a ligand bound complex to mediate transcription. Such methods and agents can be used to modulate cellular proliferation, differentiation, DNA synthesis or cell cycle distribution.

The present invention further provides methods for isolating $ER\beta_c$ or $\mathrm{ER}\beta_c$ protein isoforms (*e.g.*, ERβ-1, ERβ-2, ERβ-3 and ERβ-4) that regulate transcription. For example, ERβ-3 ligand binding partners, *e.g.*, estrogen, are isolated using the ERβ-3 protein or ligand binding portions thereof. Alternatively, for example, the DNA sequences that the ERβ-3 protein binds can be determined, for example, utilizing electrophoretic mobility shift assays (EMSA), yeast two hybrid assays, or by affinity selection and degenerate ERE consensus sequences using the DNA binding domains (DBDs) of $\mathrm{ER}\beta_c$ or its isoforms. Berkowitz and Evans, J. Biol. Chem. 267(10): 7134 (1992); Nawaz *et al.*, Gene Expr. 2(1): 39 (1992); Mosselman *et al.*, (1996).

The invention also describes methods to screen compounds that can distinguish between ERα and $\mathrm{ER}\beta_c$ and its isoforms (*e.g.*, ERβ-1, ERβ-2, ERβ-3 and ERβ-4). These methods will include methods of determining whether the compound binds and either functionally acts as an agonist or an antagonist with regard to each estrogen receptor. One method to determine whether compounds act in an agonistic or antagonistic fashion would use $\mathrm{ER}\beta_c$ in a yeast two hybrid system. Such methods have been previously employed to test the interaction of certain drugs with ERα and recognized by those of ordinary skill in the art. See Ichinose *et al.*, Gene 188: 95 (1997); Collins *et al.*, Steroids 62: 365 (1997); Jackson *et al.*, Mol. Endocrinol. 11: 693 (1997).

The biological and pathological processes that require estrogen/ERβ_c complex can be modulated further by using gene therapy methods. Additional genetic manipulation within an organism can be used to alter *ERβ_c* gene expression or the production of a ERβ_c protein. For example, an *ERβ-3* gene can be introduced into a mammal deficient for ERβ-3 protein to correct the genetic deficiency; peptide modulators of ERβ-3 activity can be produced within a target cell using genetic transfection methods to introduce into the target cells nucleic acid molecules encoding the modulators; and the *ERβ-3* gene can be introduced or deleted in a non-human mammal to produce animal models expressing *ERβ-3* gene abnormalities or delete the gene entirely (*e.g.*, knock-out mice). The latter application, *ERβ-3* transgenic animals, is particularly useful for identifying agents *in vivo* that modulate ERβ-3 activity and perhaps even other genes that encode proteins that influence ERβ-3 actions. The use of nucleic acids for antisense and triple helix therapies and interventions are also expressly contemplated.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1

Nucleotide sequence, deduced amino acid sequence and putative domain structure of the complete murine *ERβ_c* gene (*mERβ-3*)

Fig. 1 (a). Illustrates the location of each of the nine exons comprising the clone of the complete murine *ERβ_c*, *mERβ-3*, and the splicing domains that yield the different alternatively spliced isoforms of *mERβ-3*. The numbers directly above the lines signifying the exons represented by terminal nucleotides of the exon. The sizes of the nine exons in base pairs (bp) and the encoded amino acid (a.a.) sequence for each of the exons and splice variants derived from mouse ovaries is indicated. The 1,704 nucleotides of *mERβ-3* encodes a 567 amino acid protein. The letters (A through F) refer to regions of homology shared by all members of the steroid receptor super family.

Green *et al.*, Cold Spring Harbor Symposia on Quantitative Biology 51(2): 751-8 (1986). Region C corresponds to the DNA binding domain (DBD). Region E is the ligand binding domain (LBD). The newly described exon 5B lies within the LBD. Exon 5B starts with GTCCTCA and stops with CCCAAG. The shaded regions in the rendering depict the amino terminus that is included in all mERβ and rERβ isoforms and the additional exon (exon 5B) that is included in the full length (*mERβ-3*) as well as the alternative spliced rat isoform, *rERβ-4*. The deletion of exon 6 in *rERβ-4* results in a frame-shift and the juxtaposition of an in-frame stop codon causing the protein to be truncated, as indicated.

Clone mERβ-1 is 1,650 bp in length. It contains a previously undescribed 192 bp located in the 5' end of exon 1 as well as the 7 other described exons; *mERβ-1* lacks the newly described exon 5B. The isolated isoform *mERβ-2* is 1,533 bp and lacks both exon 3 and exon 5B. Isoform *rERβ-4*, isolated from rat tissue, is 1,570 bp. Although *rERβ-4* possesses the new exon 5B, it lacks exon 6. The loss of exon 6 results in a frame shift that causes translation to terminate at a stop codon located in exon 7.

Fig. 1 (b). The full length sequence of murine mERβ$_c$ (mERβ-3 clone). The additional sequence included in all mERβ clones (mERβ-1, mERβ-2), as well as the alternatively spliced rat isoform, (rERβ-4) is noted in underlined bold type. The sequence included in the ninth exon, exon 5B, is presented in lower case letters beginning at base 1,149.

Figure 2

Amino acid sequences of the alternatively spliced isoforms of the *mERβ-3*

Fig. 2 (a). Deduced amino acid sequences for alternative splice variant mERβ-1. The polypeptide sequence shared by all 3 of the alternatively spliced isoforms is indicated by the underlined sequence in bold characters. The mERβ-1 protein contains 549 amino acid residues.

Fig. 2 (b). Deduced amino acid sequences for alternative splice variant mERβ-2. The alternatively spliced mERβ-2 is 510 amino acid residues in length.

Fig. 2 (c). Deduced amino acid sequences for a rat alternative splice variant rERβ-4. This splice variant was obtained from rat ovaries. The deletion of exon 6 produces a frame shift causing a truncation that terminates 13 amino acids beyond the translated exon 5B; the resulting rERβ-4 protein likely is 414 residues long. The italicized, underlined, bold characters (residues 1-64) represent the polypeptide encoded by the novel 192 nucleotides located at the 5' terminus of exon 1. The characters indicated in bold and underlined represent the polypeptide encoded by exon 5B. The "*" refers to a translated stop codon.

Figure 3

Tissue Specific Expression of mERβ-3 Protein Detected by Western Blot

Fig. 3 (a). Using anti-peptide antibodies (Antibody 1068) raised against N-CSSEDPHWHVAQTKSAVPR-OH (the sequence encoded by exon 5B), the mERβ-3 protein was observed in a Western blot of human ovary, mouse ovary, rat ovary, ROS 17/2.8 cells, and murine primary osteoblasts protein extracts.

Fig. 3 (b). Western blot of human ovary, mouse ovary, rat ovary, ROS 17/2.8 cells, and murine primary osteoblasts protein extracts probed with antibody 1068 pre-immune sera. The protein extracts of each lane of both Figures 3(a) and 3(b) are: lane 1, human ovary; lane 2, mouse ovary; lane 3, rat ovary; lane 4, ROS 17/2.8 cells; lane 5, ROS 17/2.8 cells treated with 100 nM estradiol for 16 hours; lane 6, murine primary osteoblasts.

Figure 4

Tissue Specific Expression of rERβ DNA Detected by Southern Blot of RT-PCR Products

Fig. 4 (a). Total RNA from rat ovarian and ROS 17/2.8 cells amplified for 35 cycles using an oligo that can detect rERβ. Each lane in Fig. 4 (a) contains PCR products derived from the following types of RNA: lane 1, control, no RNA; lane 2, rat ovarian RNA (0.1 μg); lane 3, ROS 17/2.8 cells (0.1 μg); lane 4, rat ovarian RNA

control (0.1 μg), no reverse transcriptase (RT); and lane 5, ROS 17/2.8 total RNA (0.1 μg), no RT.

Fig. 4 (b). Total RNA from rat ovarian, ROS 17/2.8 cells, bone marrow RNA treated with estradiol and total RNA from primary osteoblasts in co-culture amplified for 25 cycles using an oligo derived from rERβ (Accession #U57439). Each lane in Fig. 4 (b) contains the following types and amounts of RNA: lane 1, control, no RNA; lane 2, rat ovarian RNA (2 ng); lane 3, ROS 17/2.8 total RNA (0.1 μg), lane 4, total (cultured) bone marrow RNA (0.1 μg); lane 5, total cultured bone marrow RNA (0.1 μg) where the cells had been treated with estradiol for 16 hours; lane 6, total RNA from primary osteoblasts in co-culture (0.1 μg); lanes 7-11, control reactions without reverse transcriptase (RT) for lanes 2-6, respectively.

Figure 5

Gel Shift Assay

Fig. 5(a). Gel shift analysis of mERβ-3. The receptor-DNA complex was disrupted
using the anti-peptide antibody 1067, which recognizes polypeptides encoded by exon 5B.

Fig. 5(b). Gel shift analysis of the human alpha form of the estrogen receptor (ERα). Disruption of the ERα-DNA complex was assayed using the two anti-peptide antibodies specific to exon 5B.

Both Fig. 5 (a) and (b) contain the following: lanes 1 and 2, extract alone; antibody 1067, lanes 3 and 4; antibody 1067 pre-immune serum, lanes 5 and 6; antibody 1068, lanes 7 and 8; antibody 1068 pre-immune serum, lanes 9 and 10; lanes 11 and 12 are control lanes that contain 16 μg of untransfected COS-7 nuclear extract.

Figure 6

Comparison of mERβ-3 protein with the murine ERα

The upper sequence is the protein sequence of mERβ-3, whereas the lower sequence is that of the mouse (m) mERα. The "|" between the matched sequences indicates residue identity. The ":" between the matched sequences represents similar amino acids. The "." observed in the sequences is a "gap" added by the sequence alignment program. The lines bisecting the paired sequences delineate the six domains (A-F) found in $ER\beta_c$ and ERα. There is 99% similarity and 97% identity between the C domains, which contain the DBD, of the two murine estrogen receptor subtypes. There is 79% similarity and 59% identity between the E domains, which contains the LBD.

Figure 7

Comparison between *mERβ-3* and *mERβ$_i$* nucleotide sequences

The upper paired sequence (which starts at nucleotide 151) is the nucleotide sequence of *mERβ-3*, whereas the lower sequence is the nucleotide sequence of *mERβ$_i$* published by Tremblay *et al.*, (1997). There is a one nucleotide difference between the *mERβ-3* sequence (an adenine at 1,244) and *mERβ$_i$* (a guanine at 1,009). This nucleotide difference results in an aspartic acid (D) residue in mERβ-3 and a glycine (G) residue in *mERβ$_i$*.

Figure 8

Activity of ERβ Isoforms in the presence of various estrogens

Reporter constructs expressing ERβ-1 (B1), ERβ-3 (B3), ERα (alpha), or both ERβ-1 and ERβ-3 (B1+B3) were exposed to clomiphene, diethylstilbesterol (DES), 4 OH-tamoxifen (4-OHT), or 17 β estradiol (E2). Expression was standardized to ERα response to 100 nM drug.

Figure 9

Transactivation Profiles - cV2ERE

The four panels display the ability of the different estrogen receptors to transactivate cV2ERE. The cellular response of ERα (ER Alpha), murine ERβ-1 (mER-

B1), murine ERβ-3 (mER-B3), or coexpression of both murine ERβ-1 and ERβ-3 isoforms (mER B1+B3) in COS-7 cells to E2, clomid, DES and 4-OHT were compared.

Figure 10

In situ Hybridization of Various Estrogen Target Tissues

Ovaries (upper panels), uteri (middle panels), and E-15 rat embryos were serially sectioned and probed using anti-sense (left panels) and sense (right panels) probes from ERβ and ERα. Cervical spine is shown in the lower panels.

I. General Description

Estrogen receptors are members of the nuclear hormone receptor family. Biologically, these proteins are intracellular receptors which mediate the effects of steroid hormones. Upon hormone binding, estrogen receptors control the transcriptional expression of certain hormone-responsive genes. This involves the binding of the receptors, often in homo- or heterodimeric form, to specific sequences, hormone response elements, located in the target gene promoter.

The compositions and methods of this invention provide for the screening of candidate compounds to be used to treat $ER\beta_c$ related diseases. The compositions are based on the isolation of an $ER\beta_c$ sequence, *ERβ-3*, and the three alternatively spliced isoforms, *ERβ-1*, *ERβ-2* and *ERβ-4*. Additionally, these compositions can be used to screen for $ER\beta_c$ based disease to facilitate disease prognosis and to monitor disease-related aberrant expression of $ER\beta_c$ or its isoforms.

II. Specific Embodiments

The specific embodiments disclosed in this invention relate to the isolation of the nucleic acid sequence that encodes the $ER\beta_c$ gene, *ERβ-3*. The murine (m) form of *ERβ-3* is composed of 1,704 base pairs (bp) from the ATG start codon to TGA (Fig. 1a

and b) and encodes a 567 amino acid protein; this sequence contains nine exons, including the newly described exon 5B, which is located in the region encoding the LBD. Also isolated were three alternatively spliced isoforms: *mERβ-1*, *mERβ-2* and *rERβ-4*. *mERβ-1* is 1,650 bp and encodes a 549 residue long polypeptide; ERβ-1 lacks exon 5B (see Figs. 1a and 2a). *mERβ-2* is composed of 1,533 base pairs (bp); it lacks both exon 5B and exon 3, which contains 117 bp (see Figs. 1a and 2b). The sequence encoding *rERβ-4*, an alternatively spliced isoform isolated from rat ovaries, is composed of 1,570 bp; it contains exon 5B, and the 54 bp it comprises, but exon 6, which contains 134 bp, has been deleted (see Figs. 1a and 2c).

Also described herein are methods of making and using the nucleic acid sequences corresponding to ERβ-3, its isoforms and to the proteins encoded by these nucleic acid sequences. The methods of using the nucleic acid sequences of ERβ-3 or its isoforms include determination of what tissues express $ER\beta_c$ and its isoforms (*e.g.*, ERβ-1, ERβ-2 and ERβ-4), function characterization for the proteins and nucleic acid sequences of ERβ-3 and its isoforms, development of methods to recombinantly express $ER\beta_c$ nucleic acid molecules and their associated protein products, development of an ERβ-3 reporter system, identification of ERβ-3 ligands such as estrogen that influence ERβ-3 or its isoforms and identification of compounds that modulate the influence exerted by ERβ-3 or an isoform thereof on transcriptional regulation of other genes and determining the corresponding physiological effects of such influence.

A. Isolation of the complete ($ER\beta_c$) DNA and protein

Through such methods as reverse transcriptase (RT)-PCR and/or 5' RACE (rapid amplification of cDNA ends), the complete estrogen receptor β and three isoforms were isolated. Genomic primers wee used for RT-PCR on mouse ovary RNAs to clone murein (m) *mERβ-1*, *mERβ-2* and *mERβ-3*. The sequences for *mERβ-1*, *mERβ-3* and the rat (r) isoform *rERβ-4* were obtained by 5' RACE using the Marathon system and a different set of primers. The primers and vectors chosen to isolate and clone these sequences would have been commonly known to an individual skilled in the art.

Using these techniques, a novel upstream sequence was found at the 5' end of exon 1. In mice and rats, this is an 192 bp sequence which is located at the 5' end of exon 1 of the *mERβ-3* gene, two alternatively spliced isoforms of *mERβ-3* and the alternatively spliced isoform isolated from rat ovaries (*rERβ-4*), as a result of an additional open reading frame (ORF) located upstream of the published clones. Kuiper *et al.*, (1996 and 1997); Mosselman *et al.*, (1996); Tremblay *et al.*, (1997). Further analysis by RT-PCR of mRNA derived from osteoblast and bone marrow co-cultures revealed a ninth exon, exon 5B, comprised of 54 bp and located within the LBD, as depicted in Figure 1a and b. As a result, the previously published human, rat and mouse sequences, all of which are referred to herein as $ER\beta_i$, are probably 5' truncated splice variants of this larger complete $ER\beta_c$ form, which in the murine system is *mERβ-3* (see Fig. 1a and b). The nucleic acid sequence information for *ERβ-3* predicts a 567 amino acid protein with a molecular weight of approximately 63 kDa, instead of 54 kD predicted for $ER\beta_i$.

To obtain the analogous sequences in other mammals, such as humans, the heretofore unknown *mERβ-3* gene or portions thereof can be used as probes. These probes should be of at least 18 nucleotides and preferably should be redundant for one or more sequences encoding the ERβ-3 protein; the probes are to be designed from the $ER\beta_c$ amino acid sequence and should account for the degenerate genetic code. An appropriate cDNA library, such as that for ovary, testes or prostate cells, may then be screened with the probes for cDNAs which hybridize under standard conditions to one or more of the probe compositions. For examples of such general methods, see Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL (1989). The cDNAs may then be isolated and sequenced to determine whether they code for the $ER\beta_c$ protein. In this manner, the cDNA encoding the human $ER\beta_c$ protein or other mammalian *$ER\beta_c$* genes and their respective species specific isoforms may be isolated.

A method of isolating other $ER\beta_c$ related genes is also described herein. Briefly, the nucleic acid sequences can be isolated by probing a DNA library such as that for prostate, ovary or testes, which is comprised of either genomic DNA or cDNA.

Libraries may be from either commercial sources or prepared from mammalian tissue by techniques known to those skilled in the art. The preferred cDNA libraries are human cDNA libraries which are available from commercial sources such as Stratagene.

The DNA libraries can be probed by plaque hybridization using oligonucleotide probes of at least 20 nucleic acid residues in length, which are complementary to unique sequences of murine or other *ERβ-3* genes. The preferred probes are the sequences for Primer 1 and Primer 2. The nucleic acid probes may be labeled to facilitate isolation of the hybridized clones. Labeling can be by any of the techniques known to those skilled in the art, but typically the probes are labeled with [^{32}P] using terminal deoxynucleotidyl- transferase as disclosed in Sambrook *et al.*, (1989).

Alternatively, those of skill may use polymerase chain reaction (PCR) technology to amplify nucleic acid sequences of the *ERβ-3* gene directly from mRNA, cDNA, genomic libraries or cDNA libraries. PCR or other *in vitro* amplification methods may also be useful, for example, to clone nucleic acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of *ERβ-3* DNA or *ERβ-3* mRNA in tissue samples, for nucleic acid sequencing, or for other purposes.

Appropriate primers and probes for identifying *ERβ-3* from alternative mammalian tissues are generated from comparisons of the sequences provided herein. For a general overview of PCR techniques, see PCR PROTOCOLS: A GUIDE TO METHODS AND APPLICATIONS (Innis, M, Gelfand, D., Sninsky, J. and White, T., eds.), Academic Press, San Diego (1990), incorporated herein by reference.

The present invention further provides nucleic acid molecules that encode *ERβ-1*, and the related ERβ-3 isoform proteins herein described, preferably in isolated form. As used herein, "nucleic acid" is defined as RNA or DNA that encodes a ERβ-3 polypeptide, or is complementary to nucleic acid sequence encoding such peptides, or hybridizes to such nucleic acid and remains stably bound to it under appropriate stringency conditions, or encodes a polypeptide sharing at least 75% sequence identity, preferably at least 80%, and more preferably at least 85%, with the peptide sequences.

Specifically contemplated are genomic DNA, cDNA, mRNA and antisense molecules, as well as nucleic acids based on alternative backbone or including alternative bases whether derived from natural sources or synthesized. Such a hybridizing or complementary nucleic acid, however, is defined further as being novel and nonobvious over any prior art nucleic acid including that encodes, hybridizes under stringent conditions or other appropriate stringency conditions, or is complementary to a nucleic acid encoding an ERβ-3 protein according to the present invention.

"Stringent conditions" are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl, 0.0015 M sodium titrate, 0.1% SDS at 50°C; or (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin (BSA), 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C. Another example is use of 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS. A skilled artisan can readily determine and vary the stringency conditions appropriately to obtain a clear and detectable hybridization signal.

B. Characterization of the new sequences

The complete estrogen receptor β, such as *mERβ-3*, contains nine exons. The three isoforms that have been isolated include *mERβ-1*, m*ERβ-2*, *mERβ-3* and the alternatively spliced isoform from rat ovaries, *rERβ-4*. *mERβ-1* is 1,650 bp; it contains the previously identified eight exons, lacks the new exon 5B, but contains the previously undescribed 192 bp located at the 5' end of exon 1 (see Figs. 1a and 2a). In addition to having the novel 192 bp sequence located in the 5' terminus of exon 1 and the newly described exon 5B, there is a one nucleotide difference between the sequence published by Tremblay *et al.*, (1997) and the sequence disclosed here: nucleotide 1,244 in exon 6

of the mERβ-3 sequence is an adenine whereas in the sequence by Tremblay *et al.*, (1997) it is a guanine (nucleotide 1,009).

mERβ-2 contains 1,533 base pairs (bp); *ERβ-2* lacks both exon 3 and exon 5B (see Figs. 1a and 2b). *rERβ-4* includes 1,570 bp and has exon 5B, but exon 6 is deleted (see Figs. 1a and 2c). The full length *mERβ-3* contains a previously unidentified 192 nucleotides at its 5' terminus as well as the sequences of exon 5B and exon 6. All three isoforms, as well as *mERβ-3*, contain the novel 192 bp located at the 5' terminus of exon 1.

One embodiment of this invention includes using *ERβ-3* nucleic acid sequences containing the heretofore unknown 192 bp or 54 bp (exon 5B) domains or portions thereof and placing these sequences in appropriate vectors for purposes of replication. Such vectors can then be introduced into the appropriate cell expression systems to express the proteins for use either in an assay system or to help to characterize the function of particular portions of the $ER\beta_c$ gene or its corresponding protein.

Characterization of the $ER\beta_c$ protein can be performed by creating mutants, using antibodies that recognize specific domains on $ER\beta_c$ and using polypeptide sequences to specific regions of the protein to determine their function through competition assays. This invention proposes using such techniques to characterize the specific functions of the sequences or isoforms containing the novel 192 bp and/or exon 5B (54 bp) sequences.

Another method of characterizing $ER\beta_c$ and its isoform proteins includes the use of antibodies to map out specific functional domains on the $ER\beta_c$ protein, including the LBD, the dimerization site, and the DNA binding domain (DBD) of the $ER\beta_c$ protein. Antibodies could also be utilized to determine whether the $ER\beta_c$ or its isoforms is in a functional or non-functional conformation.

C. Creating antibodies to ERβ_c protein sequences

Antibodies are useful in several areas, including determining tissue expression of ERβ_c, such as ERβ-3 or its isoforms (*e.g.*, ERβ-1, ERβ-2 or ERβ-4), and determining the functional domains of ERβ-3 or its isoforms. Once the amino acid sequence of ERβ-3 or its isoforms is known, another embodiment of this invention includes using polypeptides to create antibodies. Polypeptide sequences can be assessed using computer software to determine the antigenicity of certain polypeptide sequences for the purpose of creating antibodies to these ERβ_c specific polypeptides. Hopp and Woods, PNAS 78: 3824 (1981); Garnier *et al.*, J. Mol. Bio. 120: 97 (1978).

One antibody that has been created is an anti-peptide antibody that can distinguish between the mERβ-3 and ERβ_i. Other antibodies can be created to distinguish between the ERβ-3 isoforms, in addition to being able to distinguish between the active and inactive states of ERβ resulting from allosteric-induced ligand interactions with the receptor. The anti-peptide antibodies that distinguish between ERβ-3 and ERβ_i were prepared using conventional methods and were raised to the polypeptide sequence encoded by exon 5B with a cysteine group at the amino terminus: N - CSSEDPHWHVAQTKSAVPR - OH (Antibodies 1067 and 1068). This antibody contains all of the exon 5B polypeptide. The Jameson-Wolf antigenicity program determined that this polypeptide possesses a high degree of antigenicity. Garnier *et al.*, (1978). This program or the Hopp and Wood algorithm can also be employed to determine sequences of antigenicity in the novel amino terminus of ERβ-3 and its isoforms to develop additional antibodies.

Two other antibodies were created that recognize both ERβ-3 and ERβ_i. These antibodies (Antibodies 1069 and 1070) were created against the following sequence: N - CSSTEDSKNKESSQ - OH. This polypeptide sequence is located in the carboxy terminus of the published rat ERβ_i. Kuiper *et al.*, (1996 and 1997). Antibodies 1067 and 1068 or 1069 and 1070 were obtained from the eggs of different chickens.

An alternative method to create antibodies to ERβ-3 polypeptide sequences involves isolating ERβ-3 proteins and digesting them with various proteases. The

cleavage fragments can then be purified by size and used to raise antibodies against specific portions of ERβ-3. Finally, ERβ-3 polypeptide sequences can be created recombinantly through fusion protein techniques. ERβ-3 polypeptide sequences can be expressed by fusing the desired *ERβ-3* nucleotide sequence to, for example, the gene expressing glutathione *S*-transferase (GST). The expressed ERβ-3 polypeptide sequences created as a fusion ERβ-3/GST fusion product can then be used to create antibodies to the specific portion of ERβ-3 encoded in the *ERβ-3* containing fusion gene construct. Antibodies raised to such recombinant proteins can be either monoclonal or polyclonal and such preparation techniques are generally known.

Polyclonal antibodies 1067, 1068, 1069 and 1070 were raised in chickens. Other animals could also be utilized. Pre-immune sera was purified from 2-3 eggs collected prior to hen immunization. Immunizations were prepared with 2 mg of antigen conjugated to 2 mg Imject Keyhole limpet hemocyanin (KLH) via maleimide to the extra cysteine residue located at the amino terminus of each peptide as recommended in the manufacturer's (Pierce) instructions. The coupled carrier-antigen complex (0.5 ml) was emulsified with Complete Freund's adjuvant (0.5 ml) and 1.0 ml was used for the initial injection. The chickens were subsequently boosted every 2 weeks with coupled immunogen as described by Aves Laboratory, except that Incomplete Freund's Adjuvant was used. Six eggs were collected and the IgY was purified from the yolks. Other immunoglobulin isotypes and isotype subclasses can also be used (*e.g.*, IgG_1, IgG_2, IgM).

Monoclonal antibodies may be obtained by various techniques familiar to those skilled in the art. Briefly, spleen cells from an animal immunized with a desired antigen are immortalized, commonly by fusion with a myeloma cell (see, Kohler and Milstein, Eur. J. Immunol. 6: 511 (1976)). Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses or other methods well known to those of ordinary skill in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired antigen specificity and affinity. The yield of the monoclonal antibodies produced by such cells

may be enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate host.

Alternatively, peptide specific antibodies, such as antibodies 1067 and 1068, are prepared by immunizing suitable mammalian hosts (*e.g.*, chickens or rabbits) under appropriate immunization protocols using the peptide haptens alone, if they are of sufficient length, or if required to enhance immunogenicity, conjugated to suitable carriers. Methods for preparing immunogenic conjugates with carriers such as bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH) or other carrier proteins are well known in the art. In some circumstances, direct conjugation using, for example, carbodiimide reagents may be effective. In other instances, linking reagents, such as those supplied by Pierce Chemical Co., Rockford, IL, may be desirable to provide accessibility to the hapten. The hapten peptides can be extended at either the amino or carboxy terminus with a cysteine (Cys) residue or interspersed with cysteine residues, for example, to facilitate linking to a carrier. Administration of the immunogens is conducted generally by injection over a suitable time period and with use of a suitable adjuvant, as is generally understood in the art. During the immunization schedule, titers of antibodies are taken to determine adequacy of antibody formation. For more information, refer to Harlow and Lane, ANTIBODIES: A LABORATORY MANUAL, Cold Spring Harbor Pubs., N.Y. (1988), which is incorporated herein by reference.

While the polyclonal antisera produced in this way may be satisfactory for some applications, for pharmaceutical compositions, the use of monoclonal preparations is preferred. Immortalized cell lines which secrete the desired monoclonal antibodies may be prepared using the standard method of Kohler and Milstein or with modifications which effect immortalization of lymphocytes or spleen cells, as is generally known. Kohler and Milstein, (1976). The immortalized cell lines secreting the desired antibodies are screened by immunoassay in which the antigen is the peptide hapten or is the $ER\beta_c$ protein itself. When the appropriate immortalized cell culture secreting the desired antibody is identified, the cells can be cultured either *in vitro* or by production from ascites fluid.

The desired monoclonal antibodies are then recovered from the culture supernatant or from the ascites supernatant. Fragments of the monoclonal or the polyclonal antisera which contain the immunologically significant portion can be used as antagonists, as well as the intact antibodies. Use of immunologically reactive fragments, such as the Fv, Fab, Fab', or $F(ab')_2$ fragments, is often preferable, especially in a therapeutic context, as these fragments are generally less immunogenic than the whole immunoglobulin. The antibodies or fragments may also be produced, using current technology, by recombinant means. Regions that bind specifically to the desired regions of the receptor can also be produced in the context of chimeras with multiple species origin. Alternatively, the $ER\beta_c$ specific antibody can be humanized antibodies or human antibodies, as described in U. S. Patent No. 5,585,089 by Queen *et al.* See also Riechmann *et al.*, Nature 332: 323 (1988). Finally, given that $ER\beta_c$ protein may be involved in certain cancers, it would be useful to create bispecific antibodies capable of recognizing both the $ER\beta_c$ protein and, for example, cytotoxic T cells to facilitate the killing of tumor cells which may be useful in treating cancer. Berg *et al.*, PNAS 88: 4723 (1991).

The antibodies thus produced are useful not only as modulators of $ER\beta_c$-estrogen interaction, but are also useful in immunoassays to detect $ER\beta_c$ protein or its isoforms and for the purification of $ER\beta_c$ protein or its protein isoforms. One can use immunoassays to detect the $ER\beta_c$ protein or its alternatively spliced isoforms. Immunoassays can be used to qualitatively and quantitatively analyze the $ER\beta_c$ protein. A general overview of the applicable technology can be found in Harlow and Lane, (1988). In brief, $ER\beta_c$ protein or a fragment or isoform thereof is expressed in transfected cells, preferably bacterial cells, and purified as generally described above and in the examples. The product is then injected into a mammal capable of producing antibodies. Either monoclonal or polyclonal antibodies specific for the gene product can be used in various immunoassays; such assays include enzyme linked immunoabsorbant assays (ELISAs), competitive immunoassays, radioimmunoassays, Western blots (Fig. 3), indirect immunofluorescent assays, gel shift assays (Fig. 5) and the like.

D. Creating polypeptides that interfere with the binding domains of ERβ_c

One embodiment of this invention utilizes ERβ_c polypeptide sequences to assay their ability to interfere with ERβ_c protein mediated transcription regulation. Such interference can be created by preventing ERβ_c activating agents, such as estradiol, from binding to the ERβ_c protein. Alternatively, a polypeptide could be designed to inhibit dimerization and subsequent signaling from occurring. Such polypeptides could be created using peptide synthesizers or by creating fusion protein expressing gene constructs or other expression systems for either prokaryotic or eukaryotic cell systems.

In brief summary, the expression of natural or synthetic nucleic acids encoding mammalian $ER\beta_c$ will typically be achieved by operably linking the gene or cDNA to a promoter (which is either constitutive or inducible) and incorporating it into an expression vector. The vectors preferably are suitable for replication and integration in either prokaryotes or eukaryotes. Typical cloning vectors contain transcription and translation terminators, initiation sequences and promoters useful for regulation of the expression of the $ER\beta_c$ gene. The vectors may also comprise generic expression cassettes containing at least one independent terminator sequence, sequences permitting replication of the plasmid in both eukaryotes and prokaryotes, *i.e.*, shuttle vectors and selectable markers for both prokaryotic and eukaryotic systems.

Methods for the expression of cloned genes in bacteria are also well known. In general, to obtain high level expression of a cloned gene in a prokaryotic system, it is essential to construct expression vectors which contain, at a minimum, a strong promoter to direct DNA replication. The inclusion of selectable markers in DNA vectors transformed in *E. coli* is also useful. Examples of such markers include genes specifying resistance to ampicillin, tetracycline or chloramphenicol.

Suitable eukaryote hosts may include plant cells, insect cells, mammalian cells, yeast and filamentous fungi. In a preferred embodiment of this invention, the baculovirus/insect cell system is used for gene expression.

The protein encoded by the $ER\beta_c$ gene, which can be produced by recombinant DNA technology, may be purified by standard techniques well known to those of skill in

the art. Recombinantly produced ERβ_c can be directly expressed or expressed as a fusion protein. The protein is then purified by a combination of cell lysis (*e.g.*, sonication) and affinity chromatography. For fusion products, subsequent digestion of the fusion protein with an appropriate proteolytic enzyme releases the desired ERβ_c, its isoforms or a fragment thereof.

The purified ERβ_c, when described as "isolated" and or "substantially pure", describes a protein that has been separated from components which naturally accompany it. Typically, a monomeric protein is substantially pure when at least about 85% or more of a sample exhibits a single polypeptide backbone. Minor variants or chemical modifications may typically share the same polypeptide sequence. Depending on the purification procedure, purities of 85%, and preferably over 95% pure are possible. Protein purity or homogeneity may be indicated by a number of means well known in the art, such as polyacrylamide gel electrophoresis of a protein sample, followed by visualizing a single polypeptide band on a polyacrylamide gel upon staining. For certain purposes, high resolution will be needed and high performance liquid chromatography (HPLC) or a similar means for purification utilized.

The ERβ_c protein or its isoforms of this invention may be purified to substantial purity by standard techniques well known in the art, including selective precipitation with such substances as ammonium sulfate, column chromatography, immunopurification methods, and others. See, for instance, R. Scopes, PROTEIN PURIFICATION: PRINCIPLES AND PRACTICE, Springer-Verlag: New York (1982).

The ERβ_c polypeptides or isoform polypeptides could then be used in various assays, such as gel shift assays or yeast two hybrid systems wherein these polypeptide sequences can be tested to observe their binding ability to the hormone response elements (HRE) on DNA sequences, dimerization binding ability, and agonist/antagonist binding ability.

E. Determining tissue localization of ERβ_c or its isoforms by nucleic acid hybridization

Using portions of the newly isolated $ER\beta_c$ gene, probes can be synthesized either using polymerase chain reaction (PCR) techniques or using *in vitro* transcription, of which both techniques are known to skilled artisans. These probes, which are typically radiolabeled, can be utilized to determine which tissues express a particular $ER\beta_c$ transcript either via Northern blot analysis or dot blots of RNA samples or by Southern blots wherein the mRNA has been reverse transcribed into DNA, which is then further amplified using polymerase chain reaction (PCR) as demonstrated in Fig. 4. Southern analysis of DNA is also useful in determining whether the $ER\beta_c$ gene is present or disrupted. For example, it is known that ERα is disrupted in certain breast tumors; such information may in turn be beneficial in determining the course of chemotherapy to be utilized on a patient. Using nucleic acid sequences unique to the $ER\beta_c$, it can be readily determined what tissues express the gene.

The present invention also provides methods for detecting the presence, absence and or abnormal expression of $ER\beta_c$ gene products in a physiological specimen, as well as in other tissue samples. One method for evaluating the presence or absence of $ER\beta_c$ in a sample involves a Southern transfer and is well known to those of skill in the art (Fig. 4). Briefly, the digested genomic DNA is run on agarose slab gels in buffer and transferred to membranes. Hybridization is carried out using the probes discussed above. Visualization of the hybridized portions allows the qualitative determination of the presence or absence of the $ER\beta_c$ gene or its isoforms. Southern blotting will also distinguish, depending on the stringency conditions used for hybridization, whether the $ER\beta_c$ gene is normal or contains gene deletions or rearrangements.

Similarly, a Northern transfer may be used for the detection of $ER\beta_c$ messenger RNA (mRNA) in tissue samples of mRNA. This procedure is also well known in the art. See Maniatis *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1982). In brief, the mRNA is isolated from a given cell sample using an acid guanidinium-phenol-chloroform extraction method. The mRNA is then electrophoresed to separate the mRNA species, and the mRNA is transferred from the gel to a nitrocellulose membrane. Labeled probes are used to identify the presence or absence of the $ER\beta_c$ transcript.

An alternative means for determining the level of expression of the *ERβ_c* gene is *in situ* hybridization. *In situ* hybridization assays are well known and are generally described in Angerer *et al.*, Methods Enzymol., 152: 649 (1987). This hybridization technique has already been used to study $ER\beta_i$ expression in rat hypothalamus. Shughrue *et al.*, (1997). In an *in situ* hybridization, cells are fixed to a solid support, typically a glass slide. If DNA is to be probed, the cells are denatured with heat or alkali. The cells are then contacted with a hybridization solution at a moderate temperature to permit annealing of $ER\beta_c$ specific probes that are labeled. The probes are preferably labeled with radioisotopes or fluorescent reporters.

F. Recombinant nucleic acid molecules containing $ER\beta_c$ sequences

This invention relates to recombinant sequences that express the entire $ER\beta_c$ gene, its isoforms or portions thereof that include the newly described 5' terminus or the newly described 54 bp of exon 5B, as described in Figures 1 and 2. The invention includes all methods of expressing such recombinant constructs both in prokaryotic and eukaryotic replication systems, which would have been known to one skilled in the art.

The methods for using recombinant deoxyribonucleic acid (rDNA) or recombinant ribonucleic acid (rRNA) sequences would include sequences that form triple helixes or sequences that are antisense to the $ER\beta_c$ mRNA or isoform mRNA. Additional methods would include expressions of these recombinant nucleic acid sequences to express the encoded protein.

G. Host cells containing an exogenously supplied $ER\beta_c$ encoding nucleic acid molecule

The invention also relates to a method of introducing the recombinant full length form of $ER\beta_\sigma$ such as *ERβ-3* or one of the other isolated isoforms, *ERβ-1*, *ERβ-2* or *ERβ-4* into non-ERβ-3 expressing cells and assaying the effect said rDNA and its associated protein product have on transcriptional regulation. Cells transfected with either the full-length (*ERβ-3*) or alternatively spliced isoforms of *ERβ-3* (*e.g.*, *ERβ-1*,

ERβ-2 or *ERβ-4*) can then be utilized to assay the transfected cells' ability to form colonies in soft agar, different rates of DNA synthesis, differences in cell-cycle distribution in cells expressing different *ERβ-3* isoforms and altered morphology of the transfected cells.

The present invention further provides host cells transformed or transfected with a nucleic acid molecule encoding an ERβ-3 protein. The host cell can be either prokaryotic or eukaryotic. Eukaryotic cells useful for expression of a ERβ-3 protein are not limited, so long as the cell line is compatible with cell culture methods and with the propagation of the expression vector and expression of the *ERβ-3* gene product. Preferred eukaryotic host cells include, but are not limited to, yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell line. Particularly preferred eukaryotic host cells include insect cells. Any prokaryotic host can be used to express an *ERβ-3* encoding recombinant DNA (rDNA) molecule. The preferred prokaryotic host is *E. coli*.

Transformation of appropriate cell hosts with a rDNA molecule of the present invention is accomplished by well known methods that typically depend on the type of vector used and host system employed. With regard to transformation of prokaryotic host cells, electroporation and salt treatment methods are typically employed, see, for example, Cohen *et al.*, PNAS 69: 2110 (1972); and Maniatis *et al.*, (1982); Sambrook *et al.*, (1989); or CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, F. Ausubel *et al.*, ed. Greene Publishing and Wiley-Interscience, New York (1987). With regard to transformation of vertebrate cells with vectors containing rDNAs, electroporation, cationic lipid or salt treatment methods are typically employed, see, for example, Graham *et al.*, Virology 52: 456 (1973); Wigler *et al.*, PNAS 76: 1373 (1979); and Sambrook *et al.*, (1989).

Successfully transformed cells, *i.e.*, cells that contain a rDNA molecule of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an rDNA of the present invention can be cloned to produce single colonies. Cells from those colonies can be harvested, lysed and their DNA content examined for the presence of the rDNA using a method such as that described by Southern,

J. Mol. Biol. 98: 503 (1975) or Berent *et al.*, Biotech. 3: 208 (1985) or the proteins produced from the cell assayed via an immunological method, as discussed above.

Utilization of the full-length, fragments, or isoforms of *ERβ-3* to determine their ability to regulate the formation of colonies in soft agar is useful in assessing whether a particular isoform of the *ERβ-3* gene is responsible for cellular proliferation and/or differentiation. The ability of a particular *ERβ-3* isoform to spur proliferation and/or differentiation may in turn correspond to the gene's involvement in certain ERβ associated diseases.

Alternatively, the isoforms may be used in transfected cell lines to assay [^{3}H]-thymidine incorporation to test the effect of a particular $ER\beta_c$ isoform on DNA synthesis. Fluorescent activated cell sorting (FACS) could be utilized to determine differences in cell growth between cells bearing one isoform over another. Finally, transfected cells could be examined for morphological changes due to the expression of different $ER\beta_c$ isoforms. Once the properties of $ER\beta_c$ are determined with respect to impact on DNA expression, changes in morphology, and effects on cellular proliferation and/or differentiation, the same assays can be implemented to identify compounds that regulate the observed effects induced by isoforms of $ER\beta_c$. Identification of putative drugs, which are discussed in greater detail herein, would be valuable in modulating concentrations of $ER\beta_c$ proteins or its isoforms in diseases involving such proteins.

H. Production of $ER\beta_c$ protein using recombinant methods

This invention also describes the methods used to express the $ER\beta_c$ protein, such as by using recombinant DNA (rDNA) of the *ERβ-3* gene, such as using its novel isoforms (*ERβ-1, ERβ-2* and *ERβ-4*) or portions thereof. Methods for generating rDNA molecules are well known in the art, for example, see Sambrook *et al.*, (1989). The preferred rDNA molecules would contain an *ERβ-3* encoding DNA or a DNA encoding one of its isoforms operably linked to expression control sequences and/or vector sequences.

The choice of vector and/or expression control sequences to which one of the $ER\beta_c$ nucleic acid molecules of the present invention is operably linked depends directly, as is well

known in the art, on the functional properties desired, *e.g.*, protein expression, and the host cell to be transformed. Any vector contemplated by the present invention should be at least capable of directing replication, insertion into the eukaryote's chromosome or replicating extrachromasomally in a prokaryote, and preferably also expression of the ERβ-3 protein encoded in the rDNA molecule.

Expression control elements that are used for regulating the expression of an operably linked protein encoding sequence are known in the art and include, but are not limited to, inducible promoters, constitutive promoters, secretion signals, and other regulatory elements. Preferably, the inducible promoter is readily controlled, such as being responsive to a nutrient in the host cell's medium.

In one embodiment, the vector containing a ERβ-3 encoding nucleic acid molecule will include a prokaryotic replicon, *i.e.*, a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule extrachromosomally in a prokaryotic host cell, such as a bacterial host cell, transformed therewith. Such replicons are well known in the art. In addition, vectors that include a prokaryotic replicon may also include genes which confer such detectable markers as a drug resistance marker. Typical bacterial drug resistance genes are those that confer resistance to ampicillin or tetracycline. Vectors that include a prokaryotic replicon can further include a prokaryotic or viral promoter capable of directing the expression (transcription and translation) of the *ERβ-3* gene sequences in a bacterial host cell, such as *E. coli*. A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention. Typical of such vector plasmids are pUC8, pUC9, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA), pPL and pKK223 available from Pharmacia, Piscataway, N.J.

Expression vectors compatible with eukaryotic cells, preferably those compatible with vertebrate cells, can also be used to form rDNA molecules that contain ERβ-3 sequences. Eukaryotic cell expression vectors are well known in the art and are available

from several commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired DNA segment. Typical of such vectors are pSVL and pKSV-10 (Pharmacia), pBPV-1/pML2d (International Biotechnologies, Inc.), pTDT1 (ATCC, #31255), the vector pCDM8 described herein, and like eukaryotic expression vectors.

Eukaryotic cell expression vectors used to construct the rDNA molecules of the present invention may further include a selectable marker that is effective in an eukaryotic cell, preferably a drug resistance selection marker. A preferred drug resistance marker is the gene for which expression results in neomycin resistance, *i.e.*, the neomycin phosphotransferase (*neo*) gene as described by Southern *et al.*, J. Mol. Anal. Genet. 1: 327 (1982).

I. Diagnostic technique for measuring $ER\beta_c$ mRNA transcript levels

Another embodiment of the present invention is the use of $ER\beta_c$ nucleic acid sequences to measure changes in cells' mRNA concentrations. Methods of quantitatively and/or qualitatively assessing mRNA levels includes Northern blotting, *in situ* hybridization, nucleic acid hybridization and RT-PCR. Raval, J. Pharmacol. Toxicol. Methods 32(3): 125 (1994). One may use the coding sequence of $ER\beta_c$ or its isoforms, particularly the sequences of $ER\beta_c$, not found in $ER\beta_i$, to determine the level of mRNA present in the cell. By lysing cells obtained by a biopsy, under conditions which inhibit RNases in accordance with conventional methodologies, the mRNA may be reverse transcribed into DNA and the DNA expanded using PCR. The expanded DNA may then be quantified. Less conveniently, Northern blot analysis may be used as described above. Reverse transcription PCR (RT-PCR) has been used to ascertain specific mRNA concentrations in breast cancer cells and would be commonly known to individuals skilled in the art. See for example, Bartlett *et al.*, *Br. J. Cancer* 73(12): 1538 (1996). The benefits of using RT-PCR is that sample sizes do not have to be great to obtain valuable and sensitive results, as was observed in a study looking at mRNA levels of heart muscle biopsies. Engelhardt *et al.*, *J. Am. Coll. Cardiol.* 27(1): 146 (1996).

Determination of $ER\beta_c$ mRNA expression can also be assessed using *in situ* hybridization. This *in situ* labeling technique, which would employ labeled nucleic acid sequences capable of hybridizing to $ER\beta_c$ mRNA or its alternatively spliced isoforms and subsequent detection by a imaging device, would be useful in localizing tissues that have increased or decreased expression of $ER\beta_c$ or its isoforms' mRNA. This technique also would be commonly known to individuals skilled in the art. For example, see Guldenaar *et al.*, Brain Res. 700 (1-2): 107 (1995).

By employing any of the above diagnostic techniques, the presence and amount of transcription and expression of *ERβ-3* or its isoforms may be determined, as a measure of the expression of ERβ-3 protein, as well as other proteins for which transcription is regulated by the ERβ-3 protein. This information is related to the aggressive nature of a particular cancer, the change in the nature of the cancer in relation to treatments, such as irradiation, chemotherapy, or surgery, the metastatic nature of the cancer, as well as the aggressiveness of metastases, and the like. For example, see Maas *et al.*, Cancer Lett. 97(1): 107 (1995), which discussed changes of specific mRNA levels in breast cancer cells using RT-PCR after treatment with different anti-cancer agents. This relationship may be useful for determining the level of therapeutic treatment, monitoring the response of the tumor (or other $ER\beta_c$ related diseases) to the therapeutic treatment, and in providing a prognosis for the patient concerning the course of the disease.

J. Methods to identify agents that block $ER\beta_c$ transcriptional regulation

Another embodiment of the present invention provides methods for identifying agents that inhibit or block the association of an estrogen or estrogen-like agonists/antagonists with $ER\beta_c$ protein. For example, estrogen can be mixed with the $ER\beta_c$ protein or a cellular extract containing the $ER\beta_c$, in the presence and absence of the compound to be tested. After mixing under conditions that allow association of the estrogen or estrogen-like agonist/antagonist with $ER\beta_c$, the two mixtures are analyzed and compared to determine if the compound augmented, reduced or completely blocked the association of the estrogen or estrogen-like agonist/antagonist with the $ER\beta_c$ protein or its isoforms.

Agents that block or reduce the association of an estrogen or estrogen-like agonist/antagonist with the ERβ_c protein will be identified as decreasing the concentration of estrogen-ERβ_c binding present in the sample containing the tested compound.

The receptor protein likely must undergo allosteric change in its conformation before the estrogen-ERβ_c complex has the ability to bind to DNA. Once inside the nucleus, the activated receptor initiates transcription of genetic information from the DNA to mRNA, which is in turn a template for the linking of amino acids into proteins.

The antiestrogen effects produced by drugs such as tamoxifen (Nolvadex Registered TM) appear to be one of preventing the estrogen receptor from interacting with DNA in the nucleus to stimulate RNA and protein synthesis. This action initiates a block in the synthesis of macromolecules such as proteins, causing cell damage and the ultimate death of the cell. Antiestrogens are believed to be lipophilic molecules having a portion of the molecule which resembles naturally occurring estrogens. This portion of the antiestrogen selectively binds to the estrogen receptors. The antiestrogens, however, have a side chain arm (*e.g.*, dimethylaminophenyl ethoxy) which distorts the three-dimensional configuration of the estrogen receptor preventing translocation of the receptor to the nucleus. Morgan, U.S. Patent No. 4,732,904 (1988). Another method of determining whether candidate reagents inhibit estrogen action on the complete estrogen receptor β subtype would be by determining whether ERβ_c has undergone an allosteric transformation as a result of interacting with a candidate reagent such that ERβ_c or its isoforms can no longer combine with the native substrate, estrogen. Changes in the conformation of ERβ_c or homodimers of ERβ_c can be detected using antibodies, either monoclonal or polyclonal, to conformational epitopes that exist on ERβ_c or homodimers of the receptor. Antibodies were used to determine the functional state of ERα and a similar method could be used in determining whether compounds augment transformation into the activated allosteric conformation or inhibit the conformation all together. See Wotiz *et al.*, U.S. Patent No. 5,312,752 (1994).

Antibodies can not only be used to determine whether the ERβ_c is functionally in an active or inactive state. Antibodies could also be screened to determine whether their binding to either the ligand or to the receptor itself enhanced the binding of the ligand to the

receptor. Methods of determining said enhancement are known to the art. See Aguilar *et al.*, Mol. Cell. Biochem. 136(1): 35 (1994). Another method of determining whether a particular reagent augments or inhibits dimerization of $ER\beta_c$ or augments or inhibits $ER\beta_c$ from assuming the activated state would be to utilize a yeast two hybrid system. Yeast two hybrid systems have been successfully used to determine whether $ER\alpha$ dimerization is ligand-dependent (Wang *et al.*, J. Biol. Chem. 270(40): 23,322 (1992)); to isolate agents such as proteins or antibodies that enhance transcriptional activity of hormone receptors (Onate *et al.*, Science 270(5240): 1354 (1995)); to isolate compounds that are antagonistic to $ER\beta_c$ action in a manner comparable to what has been done with $ER\alpha$ (Ichinose *et al.*, (1997) and Collins *et al.*, (1997)); and to determine whether $ER\beta_c$ can form heterodimers in a manner analogous to what has been observed for retinoic acid receptors. See for further discussion Forman *et al.*, Cell 81(4): 541 (1995) and Walfish *et al.*, PNAS 94(8): 3697 (1997).

Another method to screen agents is to use a reporter gene such as β-galactosidase (*β-gal*) or luciferase. These transactivation experiments can be performed in yeast or in mammalian cell lines. The cells would contain $ER\beta_c$ along with an appropriate estrogen responsive element (ERE) upstream of the reporter gene (*e.g.*, luciferase or *β-gal*), such as cV2ERE. Both antagonists and agonists of ERβ can be assayed in this fashion. Gaido *et al.*, Toxicol. Appl. Pharmacol. 143(1): 205 (1997); Hafner *et al.*, J. Steroid Biochem. Mol. Biol. 58(4): 385 (1996); Muhn *et al.*, Ann. N.Y. Acad. Sci. 761: 311 (1995). Such assays can also be utilized to determine whether cross-talk occurs for $ER\beta_c$ and progesterone (PR) as has been demonstrated for $ER\alpha$ and PR. Kraus *et al.*, Mol. Cell. Biol. 15(4): 1847 (1995). Identifying novel $ER\beta_c$ responsive elements can be done rapidly using libraries of degenerate oligonucleotides. The protocol requires no purified protein and specifically selects for functional response elements. Nawaz *et al.*, (1992).

Compounds that are assayed by the above methods can be randomly selected or rationally selected or designed. As used herein, an agent is said to be randomly selected when the agent is chosen arbitrarily, without considering the specific sequences involved in the association of the estrogen or estrogen-like agonist/antagonist to the $ER\beta_c$ protein. An

example of such randomly selected agents is the use a chemical library, a peptide combinatorial library or a growth broth of an organism.

As used herein, an agent is said to be rationally selected or designed when the agent is chosen on a non-random basis which takes into account the sequence of the target site and/or its conformation in connection with the agent's action. Agents can be rationally selected or rationally designed by utilizing the peptide sequences that recognize and bind to either the estrogen or estrogen-like agonist/antagonist or to the steroid hormone binding site on the ERβ_c protein.

The agents of this embodiment can be, by way of example, peptides or other small molecules, antibodies (*e.g.*, monoclonal or polyclonal), fragments of antibodies (*e.g.*, Fv), or drugs with antiestrogenic or estrogenic activity (*e.g.*, narigenin, kaempferide, phloretin, biochanin A, flavone, ICI 182,780, raloxifene, tamoxifen, [6-hydroxy-3-[4-[2-(1-piperidinyl)ethoxy]phenoxy]-2-]4-hydroxybenzo[b]thiophene, raloxifene HCl, and ethynyl estradiol). Collins *et al.*, (1998); Palkowitz *et al.*, J. Chem. Med. 40(10): 1407 (1997). A skilled artisan can readily recognize that there is no limit as to the structural nature of the agents of the present invention. One class of compounds of the present invention includes polypeptide agents whose amino acid sequences are chosen based on the amino acid sequence of the ERβ_c LBD.

The peptide agents of the invention can be prepared using standard solid phase (or solution phase) peptide synthesis methods, as is known in the art. In addition, rDNAs encoding these polypeptides may be synthesized using commercially available oligonucleotide synthesis instrumentation and produced recombinantly using standard recombinant production systems. These rDNA molecules can then be utilized to recombinantly express polypeptides that bind to the ERβ-3 protein or its isoforms. The production using solid phase peptide synthesis is necessitated if non-recombinantly produced polypeptide sequences are to be used.

K. Administration of agents that affect ERβ_c signaling

The agents of the present invention can be provided alone, or in combination with additional agents that modulate a particular pathological process. For example, an agent of the present invention that reduces or otherwise modulates $ER\beta_c$ transcriptional regulation, by blocking estrogen or other agonist/antagonists from binding and transforming the $ER\beta_c$ protein or its isoforms into an active state, can be administered in combination with other similar agents. As used herein, two agents are said to be administered in combination when the two agents are administered simultaneously or are administered independently in a fashion such that the agents will act at the same time.

The agents of the present invention can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. The present invention further provides compositions containing one or more agents which block transcriptional regulation by the ERβ-1 protein. While individual needs may vary, determination of optimal ranges of effective amounts of each component is within the skill of the art.

In addition to the pharmacologically active agents, the compositions of the present invention may contain pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically for delivery to the site of action. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils (*e.g.*, sesame oil) or synthetic fatty acid esters (*e.g.*, ethyl oleate or triglycerides). Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Antiestrogens are typically characterized as having limited solubility, therefore the use of agents such as dimethylformamide increases

the solubility of such agonists/antagonists thus increasing their effect on, in this instance, ERβ_c or its isoforms. Sasson *et al.*, J. Steroid Biochem. 29(5): 491 (1988). Optionally, the suspension may also contain stabilizers. Liposomes can also be used to encapsulate the agent for delivery into the cell. For certain drugs such as tamoxifen, agents (*e.g.*, acetone and polyethylene glycol 4000) may be required to enhance the drug's solubility. Cotreau-Bibbo *et al.*, J. Pharm. Sci. 85(11): 1180 (1996).

The pharmaceutical formulation for systemic administration according to the invention may be formulated for enteral, parenteral or topical administration. Indeed, all three types of formulations may be used simultaneously to achieve systemic administration of the active ingredient. Suitable formulations for oral administration include hard or soft gelatin capsules, pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release forms thereof.

In practicing the methods of this invention, the compounds of this invention may be used alone or in combination with other therapeutic or diagnostic agents. In certain preferred embodiments, the compounds of this invention may be co-administered along with other compounds typically prescribed for these conditions according to generally accepted medical practice.

L. Gene therapy expression of ERβ_c

The *ERβ_c* gene, for example ERβ-3 gene and the ERβ-3 protein can also serve as a target for gene therapy in a variety of contexts. For example, in one application, *ERβ-3* deficient animals can be generated using standard knock-out procedures to inactivate a *ERβ-3* gene. In such a use, a non-human mammal (*e.g.*, a mouse or a rat) is generated in which the *ERβ-3* gene is inactivated or deleted. This can be accomplished using a variety procedures known in the art, such as targeted recombination. Once generated, the *ERβ-3* deficient animal can be used to (1) identify biological and pathological processes mediated by the *ERβ-3* gene; (2) identify proteins and other genes that interact with *ERβ-3*; (3) identify agents that can be exogenously supplied to overcome *ERβ-3* deficiency; and

(4) serve as an appropriate screen for identifying mutations within *ERβ-3* gene that increase or decrease activity.

In addition to animal models, human *ERβ*$_c$ deficiencies or mutations can be corrected by supplying to a patient a genetic construct encoding the necessary ERβ$_c$ protein. A variety of techniques are presently available, and others are being developed, for introducing nucleic acid molecules into human subjects to correct genetic deficiencies and mutations. Such methods can be readily adapted to employ the *ERβ*$_c$ encoding nucleic acid molecules of the present invention.

In another embodiment, genetic therapy can be used as a means for modulating an ERβ$_c$ mediated biological or pathological process. For example, during osteoporosis, it may be desirable to introduce into the patient a genetic expression unit that encodes a modulator of ERβ$_c$ mediated transcriptional regulation, such as a nucleic acid molecule that is antisense to the ERβ$_c$ mRNA. Alternatively, tissue specific co-activators or co-repressors could be identified and introduced into a recipient to augment modulation of ERβ$_c$ or its isoforms. Such a modulator can either be constitutively produced or inducible within a cell or specific target cell. This allows a continual or inducible supply of a therapeutic agent within the patient.

M. Conformational analysis of ERβ$_c$ using antibodies

Using site-specific polyclonal and monoclonal antibodies against the DNA binding domain (DBD) of the ERβ$_c$ protein, one can determine the active state of the protein. On this basis, the user is able to determine whether the DBD of ERβ$_c$ is present in a functional or non-functional altered state and whether the ERβ$_c$ protein has been activated or not. The invention, therefore, includes specifically prepared immunogens, polyclonal antisera and monoclonal antibodies which bind specifically to the DBD of ERβ$_c$ or its isoforms, and immunoassays employing these site-specific antibodies with cellular samples on a functional and correlative test basis, as described above. Similar procedures and methods have been utilized in determining whether ERα is in its active or inactive state. Wotiz *et al.*

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods; additionally, all the preceding description involving *ERβ-3* or, alternatively spliced isoforms of the complete gene, (*e.g.*, *ERβ-1*, *ERβ-2* and *ERβ-4*) can be applied to their analogs in other mammalian species. The following working examples therefore, specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure. Other generic configurations will be apparent to one skilled in the art.

EXAMPLES

Example 1

Cloning of the complete murine *mERβ-3* cDNA

The *mERβ-3* clone was twice isolated using two separate procedures: (1) reverse transcriptase PCR (RT-PCR) of mRNA, and (2) amplification from a mouse embryonic stem (ES) cell genomic DNA library. A mouse ES cell genomic DNA library was screened using a cDNA probe and RT- PCR. The oligonucleotides chosen, corresponded to regions in the D and E domains of rat $ER\beta_i$ published by Kuiper *et al.*, (1996). These oligonucleotides were: 5'- ATG ACA TTC TAC AGT CCT GCT GTG ATG-3' (Primer 1) and 5'-GAA GTG AGC ATC CCT CTT TGC GTT TGG-3' (Primer 2). Using these oligonucleotides five clones were obtained. Two primers were chosen in these genomic DNAs, one around the first ATG, which is 192 bp upstream from the published ATG (Kuiper *et al.*, (1996); Mosselman *et al.*, (1996); and Tremblay *et al.*, (1997)), 5'-TCT CTG AGA GCA TCA TGT CC-3' (Primer 3), and one around the TGA, 5'-CAG CCT GGC CGT CAC TGT GA-3' (Primer 4). The RT-PCR was performed on 10 and 100 ng samples of mouse ovary RNA using the Titan™ RT-PCR System of Boehringer Mannheim according to manufacturer's instructions. The amplified products obtained using Primers 3 and 4 underwent a second amplification using: 5'-TGC TCT AGA CCA CCA TGT CCA TCT

GTG CCT CT-3' (Primer 5) and 5'-CCG GAA TTC TCA CTG TGA CTG GAG GTT CTG 3' (Primer 6). The products obtained using Primers 6 and 7 were then inserted into Bluescript® vector. The same conditions were used to clone *mERβ-1*, *mERβ-2* and *mERβ-3*.

The *mERβ-3* clone was also isolated from mRNA using the Marathon RT-PCR system from Clontech. For 5' RACE, poly A+ RNA was prepared from total RNA derived from mouse ovaries according to the methods described in Sambrook *et al.*, (1989). Approximately 0.5 μg of the poly A+ RNA was reverse transcribed using 200 U Superscript II exogenase- (exo-) using the Marathon cDNA synthesis primer, 5'-TTC TAG AAT TCA GCG GCC GC(T_{30})-3', according to manufacturer instructions (GIBCO). The second strand synthesis and all subsequent steps, except PCR, were performed according to the conditions described by Marathon. The cDNA (0.5 μl of a 10 μl reaction) was then amplified using the Marathon adaptor primer, 5'-CCA TCC TAA TAC GAC TCA CTA TAG GC-3', with one of two gene specific reverse primers in the presence of Advantage *Taq* polymerase: 5'-GCA GTA GCT CCT TCA CCC G-3' (Primer 7) or 5'-GCA CTT CAT GCT GAG CAG-3' (Primer 8). The following four step thermocycling program was used to amplify the two products: (1) 5 cycles, 30 sec at 94°C, 4 min at 72°C; (2) 5 cycles, 30 sec at 94°C; (3) 25 cycles , 4 min at 70°C; and (4) 20 sec at 94°C, 4 min at 68°C. Single, predominant amplicons corresponding to the 5' end of the cDNA were then digested with restriction enzymes, cloned and sequenced. The clone was then inserted into a Bluescript® vector as described above.

Once the *mERβ-3* gene was cloned, both the nucleic acid and the amino acid sequences were deduced for the complete estrogen receptor β sequence (see Figs. 1a and b). In addition to having the novel 192 bp sequence located in the 5' terminus of exon 1 and the newly described exon 5B, nucleotide 1,244, an adenine, in exon 6 of the *mERβ-3* sequence differs from the guanine (nucleotide 1,009) found in the sequence by Tremblay *et al.*, (1997). The *mERβ-3* gene is 1,704 nucleotides long and encodes a 567 amino acid protein. Using these techniques, it was ascertained that the 5' end of *mERβ-3* included an additional open reading (192 bp) frame as well as a ninth exon, exon 5B (54 bp); both of these

sequences are not found in ERβ_i. Kuiper *et al.*, (1996); Mosselman *et al.*, (1996); and Tremblay *et al.*, (1997). Exon 5B is located in the ligand binding domain (LBD) of *mERβ-3* and likely plays a significant role in mERβ-3 function.

Example 2

Isolation of three alternatively spliced isoforms

In addition to the complete murine estrogen receptor β gene sequence, *mERβ-3*, two other alternatively spliced murine forms of *mERβ-3* were identified (*mERβ-1* and *mERβ-2*,) as well as a fourth alternatively spliced isoform isolated from rat ovaries, *rERβ-4*. The first alternatively spliced form of *mERβ-3*, *mERβ-1*, contains the novel 192 bp at the 5' terminus of exon 1, but lacks the 54 bp of exon 5B; it is 1,650 nucleotides in length and putatively encodes a 549 amino acid long polypeptide (Fig. 2a). Preliminary data indicates that the *mERβ-1* isoform may be more active than the full length *mERβ-3*. The *mERβ-1* isoform was isolated using both methods described for the isolation of *mERβ-3*.

Isoform *mERβ-2* is composed of 1,533 bp, which would encode 510 amino acids (Fig. 2b); *mERβ-2* lacks exon 3, which contains 117 bp. The *mERβ-2* isoform was isolated only from the mouse ES cell genomic library.

Isoform *rERβ-4* was obtained from rat (r) ovaries whereas *mERβ-1* and *mERβ-2* as well as the full length *mERβ-3* were obtained from mouse (m) ovaries; it is 1,570 nucleotides in length and contains exon 5B, but exon 6 is deleted. Exon 6 is comprised (as shown in Fig. 1a) of 134 bp. The putative protein product of *rERβ-4* would be 414 amino acids (Fig. 2c).

All the nucleic acid sequences discussed relate to the coding regions and sequences for the corresponding mRNAs would be longer in both their 5' and 3' regions. It is likely that the published incomplete estrogen receptor β genes (*ERβ$_i$*) isolated from human, rat and mouse libraries are splice variants of this complete form, which in mice is *mERβ-3*, and contains the 54 bp of exon 5B and the 192 bp located at the 5' terminus of exon 1. Mosselman *et al.*, (1996); Kuiper *et al.*,(1996); and Tremblay *et al.*, (1997). All four

sequences contain the 192 bp located at the 5' terminus of exon 1 and not described in the previously published sequences. *Id.* A sequence similar to the novel 192 bp region located in the 5' terminus of exon 1 may also exist in human ERβ_c and its isoforms.

The alternatively spliced isoforms (*e.g.*, *mERβ-1*, *mERβ-2* and *rERβ-4*) of the full length murine ERβ_c gene, *mERβ-3*, were twice isolated using the same two different procedures used to acquire *mERβ-3*. The primers used in both Examples 1 and 2 were selected based on the assumption that variants, if any, would occur within the boundaries of these selected primers. Historically, similar primers have produced analogous results with ERα.

Once the isoforms were isolated the DNA sequences could be sequenced and the amino acid sequence encoded by each could be determined. The proteins for the three alternatively spliced isoforms are shown in Figures 2a, 2b and 2c.

Example 3

Tissue specific expression of mERβ-3 protein using Western Blotting

Anti-peptide antibodies raised against a sequence specific to the mouse ERβ_c (mERβ-3) specifically recognized a protein of 64 kDa in ovary and in bone, as well as in other tissues. Two anti-peptide antibodies were raised in chickens to N-CSSEDPHWHVAQTKSAVPR-OH (Antibodies 1067 and 1068); this polypeptide is encoded by exon 5B and recognizes mERβ-3 as well as the isoforms that express the exon 5B coding region. Antibody 1067 and 1068 were obtained from the eggs of two different chickens, as were antibodies 1069 and 1070. These antibodies recognize the protein produced by *mERβ-3*, but not the ERβ_i protein discovered by Kuiper *et al.*, (1996 and 1997), which lacks exon 5B.

Total proteins (60 μg) obtained from ovarian tissue or bone tissue samples were resolved by electrophoresis in 10% SDS acrylamide gels; the gels were electrophoresed for 16 hours at 40 V. The proteins were transferred from the gels onto nitrocellulose membranes; the transfer was done for 4 hours at 100 mA. The blots were probed using a 1:1,000 dilution of the chicken antisera to mERβ-3 (Antibody 1068) in conjunction with a 1:1,000 dilution of a secondary antibody conjugated to horseradish peroxidase (Promega).

The proteins were visualized using the ECL chemiluminescent substrate, and exposed to film (BMR film, Kodak) for one minute.

Figure 3 shows the results of the Western blot obtained using Antibody 1068, which detects the polypeptide encoded by exon 5B. Total protein (60 μg) was resolved by electrophoresis. The proteins were transferred to nitrocellulose membrane and probed with a 1:1,000 dilution of Antibody 1068 (Fig. 3a). Figure 3(b) is the blot probed with antibody 1068 pre-immune sera. The protein extracts of each lane of both Figures 3(a) and 3(b) are: lane 1, human ovary; lane 2, mouse ovary; lane 3, rat ovary; lane 4, ROS 17/2.8 cells; lane 5, ROS 17/2.8 cells treated with 100 nM estradiol for 16 hours; lane 6, murine primary osteoblasts. The antibody specifically recognizes a 64 kDa protein, which closely approximates the predicted size of mERβ-3. The question mark refers to a protein migrating at approximately 58 kDa that may be immune specific but is otherwise unidentified. ROS 17/2.8 cells are a line characterized by Gideon Rodan; it is a rat osteoblast-like osteosarcoma cell line (ROS).

Example 4

Tissue specific expression of rat *ERβ* determined by Southern Blotting of RT-PCR Products

Cell expression of rat (r) *ERβ* (*rERβ*) mRNA was examined Southern blotting of RT-PCR products. Total RNA (2-100 ng) obtained from rat ovary, rat total bone marrow (100 ng), and ROS 17/2.8 cells (100 ng) were reverse transcribed using 200 U of Superscript (exo-) reverse transcriptase (Gibco-BRL) and 100 pmol random hexamer probe according to the manufacturer's recommended conditions. ROS 17/2.8 cells are a rat osteoblast-like osteosarcoma cell line (ROS). The rat cDNA was then amplified by PCR in 100 μl reactions using 2 U *Taq* polymerase and 1 μM 5'-GTC AAG TGT GGA TCC AGG-3' (Primer 9; beginning at base 924 of Accession U57439 and corresponding to base 700 of *mERβ-3*) and 5'-GCT CAC TAG CAC ATT GGG-3' (Primer 10; beginning at base 1,130 of $rER\beta_i$ by Kuiper *et al.*, Accession U57439, and corresponding to base 906 of mERβ-3) per each individual reaction. Products were amplified using 25-40 cycles of the following amplification program: 90°C x 1 min; 55°C x 45 sec; 72°C x 2 min. The product was allowed to be extended at 72°C x 5 min at the end of the program.

Following amplification, the PCR products were resolved in a 4% NuSieve agarose (FMP)/TBE gel; the DNA was transferred to nylon membranes (Boehringer Mannheim) and cross-linked by UV irradiation for Southern analysis. Ten pmol of an oligonucleotide internal to the predicted amplicon 5'-AGC AGG TAC ACT GCC TGA GCA AAG CCA AGA-3' (Primer 11; beginning at base 991 of Accession U57439 and corresponding to bases beginning at 767 of *mERβ-3*) was end-labeled using T4 polynucleotide and used to probe the immobilized DNA amplicon. Following pre-hybridization at 58°C in Quick-Hyb hybridization solution (Stratagene), the probe was added and allowed to hybridize for 1 hr. The blot was then washed twice with 2x SSC containing 0.1% SDS at room temperature, and then twice with 0.1 X SSC and 0.1% SDS at 58°C. The blot was then exposed to film.

Figure 4 is an autoradiograph of Southern blot of rat ERβ (rERβ) products amplified by RT-PCR. Total RNA from a variety of tissues was reverse transcribed, amplified by PCR, transferred to nylon membranes and probed using a ^{32}P labeled mERβ-3 oligonucleotide. Figure 4 (a) was amplified for 35 cycles. Each lane in Figure 4 (a) contains the following types and amounts of RNA: lane 1, control, no RNA; lane 2, rat ovarian RNA (0.1 μg); lane 3, ROS 17/2.8 cells (0.1 μg); lane 4, rat ovarian RNA control (0.1 μg), no reverse transcriptase (RT); and lane 5, ROS 17/2.8 total RNA (0.1 μg), no RT.

Figure 4 (b) is a Southern blot of total RNA. The ERβ products were amplified for 25 cycles by RT-PCR. Each lane in Figure 4 (b) contains the following types and amounts of RNA: lane 1, control, no RNA; lane 2, rat ovarian RNA (2 ng); lane 3, ROS 17/2.8 total RNA (0.1 μg), lane 4, total (cultured) bone marrow RNA (0.1 μg); lane 5, total cultured bone marrow RNA (0.1 μg) where the cells had been treated with estradiol for 16 hours; lane 6, total RNA from primary osteoblasts in co-culture (0.1 μg); lanes 7-11, control reactions for lanes 2-6, respectively.

Discrimination analysis for the relative expression of $ERβ_c$ isoforms may be done utilizing random primers and reverse transcriptase (RT) to synthesize the cDNA from various rat or mouse or other mammalian tissues. The cDNAs so obtained are then amplified by PCR using the completely homologous rat and mouse primers 5'-GTC AAG TGT GGA TCC AGG-3' (Primer 9), which corresponds to base 700 of mERβ-3 or base 924 of *Rattus norvegicus* estrogen receptor β mRNA, accession U57439 (Kuiper *et al.*, 1996),

and 5'-GCA CTT CAT GCT GAG CAG-3' (Primer 8) corresponding to base 1,554 of mERβ-3 and 1,724 of accession U57439. Following amplification, the PCR products are purified and digested with *Fsp I*, a restriction endonuclease with a consensus site within exon 5B (TGCGCA at base 1,176 of mERβ and also present in rERβ-4). Digestion of the mouse or rat amplicons bearing the exon 5B sequence thus yields smaller products. The digested PCR products are resolved by agarose gel electrophoresis, transferred to nylon membranes, and probed with complementary oligonucleotide probes specific to either rat or murine sequences, or both. The specific sizes of the hybridized DNA present determines what isoform is present in a particular tissue or cell sample. Additionally, the intensity of the band allows quantitation of the relative abundance of the isoform(s) in a particular sample.

Example 5

Gel Shift Assays

Gel shift analysis of mERβ-3 is demonstrated in Figure 5(a). The results obtained by the mERβ-3 gel shift (Fig. 5a) were compared to that obtained for the human estrogen receptor alpha (ERα) form, as displayed in Fig. 5(b). The receptor-DNA complexes formed were then disrupted using anti-peptide antibodies directed toward the novel exon 5B (Antibodies 1067 and 1068). Nuclear extracts (16 μg) derived from COS-7 cells transfected with expression plasmids containing mERβ-3 (Fig. 5a) or human alpha estrogen receptor (pHEGO) (Fig. 5b) were incubated with 5 fmols of 27 base pair perfect ERE end-labeled with ^{32}P isotope. The description for the lanes in both figures are the same. The lanes for both Fig. 5 (a) and (b) contain the following: lanes 1 and 2, extract alone; antibody 1067, lanes 3 and 4; antibody 1067 pre-immune serum, lanes 5 and 6; antibody 1068, lanes 7 and 8; antibody 1068 pre-immune serum, lanes 9 and 10; lanes 11 and 12 are control lanes that contain 16 μg of untransfected COS-7 nuclear extract.

Example 6

Relative Affinities of Various Estrogens for ER Subtypes

The experimental data displayed in Table I demonstrates the different affinities that various estrogens have for the ERβ subtypes (*e.g.*, ERα, ERβ-1 and ERβ-3).

E2 binding affinity was determined by incubating transfected COS-7 cell cytosol with different concentrations of [^{3}H]-E2 (0-200nM) and with or without unlabeled E2 500X for 4 h at 4°C in 40 mM Tris HCl pH 7.4, 150 mM KCl, PMSF 0.1 mM, DTT 2 mM. COS-7 cells were transfected as described in Example 7. Bound receptor was separated by the hydroxy apatite method (Obourn *et al.*, Biochemistry 32(24): 6229-6236 (1993)) or the ligand was removed by the dextran coated charcoal method (Garcia *et al.*, Mol. Endocrinol. 6(12): 2071-2078 (1992)), and bound hormone measured by liquid scintillation counting. Dissociation constants (kd) were obtained by Scatchard plots. Similarly, the relative affinities of different estrogenic ligands were determined using 1 nM receptor and 5 nM [^{3}H]-E2 with or without various concentrations of the described competitor steroids. The concentration of ligand necessary to displace 50% of the bound, labeled [^{3}H]-17β-estradiol from the receptor, was used in the denominator to express the values shown in table (with a constant value of 1 nM in the numerator).

The differences in the relative affinities (results of the IC-50 experiments) show that the mERβ-1 and mERβ-3 receptors have different affinities for different ligands. This suggests that the transcriptional responsiveness to different ligands is a function of both the expression pattern of the receptor sub-type, and the estrogenic ligand used to stimulate transcription.

Table I shows the different affinities of estrogens to human ERα, mouse ERβ-1 and mouse ERβ-3 (which contains exon 5B). As indicated, the affinity of the different estrogens varies as to the receptor. The larger the number, the greater the affinity the estrogen has for the estrogen receptor target. Diethylstilbestrol (DES) has a greater affinity for the ERβ isoforms than for ERα.

TABLE I. Relative Affinities of Various Estrogens for ER Subtypes

Estrogen	ERα Human	ERβ-1 Mouse	ERβ-3 Mouse
17 β Estradiol (standard)	100 Kd = 0.40 nM	100 Kd = 0.57 nM	100 Kd = 7.14 nM
Diethylstilbestrol	283	432	1009
Ethynyl Estradiol	216	34	189
RU 58668	208	111	475
Raloxifene	143	22	279
RU 39411	126	147	900
Moxestrol	87	16	132
4 OH-Tamoxifen	49	94	291
Estriol	5	14	38
17-α-estradiol	2.5	0.8	7
Tamoxifen	2	5	10
Estrone	1.3	2.3	21.5

The method used in this experiment can be utilized for screening reagents with different affinities for each of the ERβ isoforms and comparing them to the ERα for determination of the affinity a particular drug may have for the other estrogen receptor proteins and their isoforms.

Example 7

Transactivation Profiles of ERβ-1 and ERβ-3 Isoforms

This experiment assessed the effect of ERβ-1 and ERβ-3 isoforms when expressed both individually and when expressed together as compared to the effect of ERα.

The ability of estrogens to stimulate transcription via an estrogen response element (ERE) functionally linked to tk-CAT (a construct described by Metzger *et al.*, J. Biol. Chem. 270(16): 9535 (1995)) was measured by transient transfection of the expression vectors for mERβ-1 and mERβ-3 in COS-7 cells. For transfection, COS-7 cells were seeded into six-well plates in phenol red free, low glucose DMEM. At approximately 50-80% confluency, the cells were transfected using lipofectamine according to the manufacturer's instructions (GIBCO-BRL). The expression constructs were transfected with a total of 2 μg DNA containing 500 ng of reporter, 100-500 ng expression plasmid, and the remainder (1-1.4 μg) as pBluescript as a carrier DNA. After 24 h, the cells were washed with DMEM and replaced with fresh medium containing drug (17-β estradiol, 4-hydroxy tamoxifen, clomiphene or DES at 1-300 nM concentrations) or vehicle (ethanol). After 24 h the cells were lysed, and the CAT activity determined by liquid scintillation counting of converted chloramphenicol (as described in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY). For co-transfection of both mERβ-1 and mERβ-3 isoforms, equivalent amounts of expression constructs were transfected (usually 100 ng each) (Fig. 8). To analyze the effect of other drugs on transcription, similar experiments were performed as described above, with the exception that drug concentrations were varied (Fig. 9).

The results of transfection analysis (Fig. 8) show that mERβ is capable of stimulating transcription from a reporter containing a canonical responsive element. The mERβ-1 can stimulate transcription to approximately 50-70% of that observed in similar cells transfected with the ERα construct, pHEGO, at estradiol concentrations of 100 nM. The mERβ-3 isoform is capable of stimulating transcription to only approximately 40% of that observed in pHEGO at the 100 nM drug concentration.

As can be seen in Figures 8 and 9, the mERβ-1 responsiveness is similar to that observed in cells transfected with pHEGO, which encodes ERα. mERβ-3 only stimulated transcription at very high estrogen concentrations (100-200 nM). By contrast, when both receptors are co-expressed together, the magnitude of the response is ablated, showing that mERβ-3 functions as a dominant negative modulator of the action of mERβ-1. (Similar

results were corroborated in an article published by Maruyama *et al.*, Biochemical Biophysical Research Communications 246(1):142-147(1998)).

The results in Figure 8 show that mERβ-1 has a transactivation profile similar to ERα when exposed to E2, clomiphene (clomid), diethylstilbestrol (DES) and 4-OHT. The mERβ-3 isoform has a decreased ability to transactivate cV2ERE as compared to either ERα or mERβ-1. However, the transactivation activity is reduced when the isoforms are co-expressed (Fig. 8, panel indicated as mER B1+B3). The assay utilized in this example can be similarly used to determine what agents can modulate homodimers of ERβ isoforms, as well as heterodimers of the ERβ isoforms or heterodimers composed of ERβ and ERα isoforms.

Figure 9 demonstrates that ERβ-1 (displayed as B1 in Fig. 9) and ERβ-3 (B3) both possess similar activity when exposed to clomiphene, DES, 4-OHT, and E2. However when ERβ-1 and ERβ-3 are co-expressed in a reporter system, their activity is down regulated as compared to individual expression of the ERβ isoforms or to ERα. This assay system can be utilized to screen other estrogens or compounds that modulate the activity of the various ERβ isoforms.

Example 8

In Situ Hybridization of various tissues

In situ hybridization analysis was performed using anti-sense cRNA probes to both the ERα and ERβ to localize the message for each of the ER subtypes. The tissue was treated with 0.1 M TEA, pH 8.0, plus 0.25% acetic anhydride for 10 min at room temperature, rinsed three times in 2X SSC, dehydrated through a series of alcohols and air dried. cRNA riboprobes corresponding to the ERα or ERβ-3 isoforms were prepared and used to probe tissue sections. The hybridization solution was removed, the sections washed and air dried. For riboprobes, an 801 base pair insert corresponding to the ligand binding domain of the mERβ-1 plasmid (bases 931-1731 of the rat sequence) was linearized using the restriction enzyme *ApaLI* and transcribed using RNA polymerase *in vitro* in the presence of [^{35}S]-UTP and [^{35}S]-CTP according to methods of Goldstein *et al.*, Neuroscience 71(1): 243 (1996). The riboprobes were purified by ethanol precipitation, and the dried tissue

sections hybridized with probe in hybridization buffer overnight at 55°C. The hybridization solution was removed, the sections were incubated briefly with RNase, then washed, dehydrated, and air dried. The dried sections were exposed to film for normalization of subsequent exposure times and dipped in NTB3 emulsion to determine the cellular and anatomical localization of each mRNA.

The results demonstrate abundant and wide spread distribution of the ERβ message within the developing ovarian follicle (Fig. 10, top panel) and in the lung, kidney cortex, and specific regions of the brain (not shown). The pattern of distribution of ERα was quite different and was highly expressed only in the uterus (Fig. 10, middle panel), in the medullary regions of the kidney (not shown) and specific regions of the brain. Preliminary data also indicated that ERβ is expressed in ossification center that appear to correspond with mesenchymal condensation zones in developing rat bone (12 days), especially in the spine (Fig. 10).

The ERβ message is observed in developing Graffian follicles (GA), but not in resorbing follicles (FA) undergoing atresia (Fig. 10, top panel, antisense). In the ovary, the ERα message receptor was only abundant within the uterine tube (not shown). ERα mRNA was observed to be widely expressed throughout the uterus (Fig. 10, middle panel, antisense). In the cervical spine, the ERβ mRNA was localized to zones of mesenchymal ossification (M) similar to the expression patter of Osf/Cbfa1, an osteoblast differentiation protein (Fig. 10, bottom panel, antisense, arrows). Controls corresponding to serial sections hybridized using sense riboprobe controls are also shown in the panels on the right.

<u>Example 9</u>

<u>Methods of Screening for Drugs</u>

A. Phosphorylation of ERβ. Most of the members of the steroid receptor superfamily, including ERα, undergo post-translational modifications (*e.g.*, phosphorylation) as a function of their basal state or in response to ligand binding. With ERα, there are a variety of sites on the molecule that are phosphorylated in response to ligand binding. Post-translational modification of mERβ or human ERβ can be accomplished using the same methods as previously utilized for ERα. Methods of

analyzing phosphorylation include transient or stable expression of the various cDNA constructs in COS-7 cells, or by immunoprecipitation of [^{32}P]-labeled ERβ from cells metabolically labeled with [^{32}P]-orthophosphate. Tryptic maps from ligand stimulated or unstimulated cells can be obtained using ERβ proteins isolated by immunoprecipitation of the mERβ or human ERβ molecule using our antibodies (*e.g.*, directed towards products of exon 5B such as the antibody used to obtain Fig. 3) or commercially available antibodies. For studies performed using *in vitro* transient transfection, a triple-myc tag or GST tag can also be linked to the carboxyl or amino termini by cloning the appropriate coding sequence into the expression plasmid. The expressed (phosphorylated) protein can then be immunoprecipitated using a very reliable, and commercially available anti-myc antibody (if using the triple-myc tag) or anti-GST antibodies. In addition, exon 5B amino acid residues can be substituted with other residues to prevent phosphorylation. In contrast to either ERα or mERβ-1, exon 5B, which is unique to mERβ-3, is located within a region of the molecule that otherwise is extremely hydrophobic. The exon 5B region, however, is unusually hydrophilic and contains a consensus casein kinase II (CKII) phosphorylation site (VLDRSSEDP) that arises as direct consequence of the location of the exon 5-exon 5B-splice junction. Many of the steroid receptors, including the ERα subtype, are phosphorylated on CKII sites. The serines present in the portion of ERβ encoded by exon 5B can be substituted with alanine residues (or other uncharged amino acids) or with residues which mimic constitutively phosphorylated molecules (*e.g.*, aspartic acid residues). Such forms of ERβ can be utilized in screening and isolating drugs which modulate the activity of the various ERβ isoforms. Alternatively, these mutant forms of ERβ or polypeptide fragments containing this region can themselves be tested for agonist or antagonist activity in the ERβ signal pathways.

B. Domain Switching. The amino terminus of the ERα contains an autonomous transcriptional activity (AF-1) that is only fully active when "integrated" with the ligand-dependent transcriptional domain (AF-2) present within the ligand binding domain of the ERα molecule. While these domains have yet to be described for the ERβ molecule, the high degree of sequence homology at the protein level between ERα and ERβ molecules logically suggests that ERβ is similarly organized. Many of these domains have been

identified and characterized using portions of the ligand binding domain (LBD) fused to convenient and reliable epitope tags, such as GST and myc. Such constructs can then be utilized to identify, in whole cell lysates or other expression models such as expression libraries, proteins that functionally alter the transcriptional responsiveness of the ER complex. We postulate that specific integrator molecules may be found using the LBD of mERβ-3 fused to such convenient epitope tags as probes for protein-protein interactions. Such proteins can then alter the transcriptional responsiveness of the functional ER complex, (defined as the homo-dimers of ERβ3 with ERβ3 or hetero-dimers of ERβ3 with ERβ1, or ERβ3 with ERα) portion of the amino terminus fused with such epitope tags as probes for proteins that interact with the ER-complex. These complexes in turn can be used in drug screening assays to identify drugs which modulate ERβ isoform activity. Alternatively the complexes themselves may be used to regulate pathways mediated by estrogen receptors.

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WHAT IS CLAIMED:

1. An isolated nucleic acid molecule that encodes mammalian *ERβ$_c$*, and its allelic variants or isoforms.

2. The isolated nucleic acid molecule of claim 1 that encodes human *ERβ$_c$*, and its allelic variants or isoforms.

3. The isolated nucleic acid molecule of claim 1 that encodes murine *mERβ-1*, *mERβ-2* or *mERβ-3* or rat *rERβ-4*.

4. The isolated nucleic acid molecule of any of claims 1-3, wherein said nucleic acid molecule is operably linked to one or more expression control elements.

5. An isolated nucleic acid molecule of any of claims 1-3 or a portion thereof, wherein said nucleic acid molecule hybridizes to a nucleic acid molecule of claims 1-3 under conditions of sufficient stringency to produce a clear signal.

6. A host transformed to contain a nucleic acid molecule of claim 5.

7. The host of claim 6, wherein said host is selected from the group consisting of prokaryotic hosts and eukaryotic hosts.

8. A method of producing mammalian *ERβ$_c$*, allelic variants or fragments thereof, comprising the step of culturing a host of claim 7 under conditions in which the ERβ$_c$ protein or polypeptides thereof are expressed.

9. An isolated protein comprising the amino acid sequences depicted in Figures 1b, or 2a, or 2b, or 2c, or portions thereof.

10. An isolated antibody that binds to the protein of claim 9.

11. The antibody of claim 10, wherein said antibody is selected from the group consisting of a monoclonal antibody, a humanized antibody, a human antibody, a bispecific antibody, a chimeric antibody, and an antibody fragment such as Fv, Fab, Fab', or $F(ab')_2$.

12. An antibody of claim 11, wherein said antibody that recognizes and binds to N-CSSEDPHWHVAQTKSAVPR-OH or N-CSSTEDSKNKESSQ-OH or polypeptide sequences that contain these sequences or portions of these sequences.

13. A method for blocking the interaction of an estrogen selected from the group consisting of physiological estrogens, stilbene estrogens or triphenylethylene (anti)estrogens with the protein of claim 9 comprising the step of contacting said protein with an agent that blocks the binding of the protein to physiological estrogens, stilbene estrogens or triphenylethylene (anti)estrogens.

14. The method of claim 13, wherein said agent blocks the binding of physiological estrogens, stilbene estrogens or triphenylethylene (anti)estrogens to said protein by selectively binding to a hormone binding domain, to a DNA binding domain, or to a dimerization domain of the protein of claim 9.

15. The method of claim 14, wherein said agent is selected from the group consisting of a fragment of said protein, an antibody that binds to said protein, and an antibody fragment, such as Fv, Fab, Fab', or $F(ab')_2$ that binds to said protein, and a humanized antibody, human antibody, bispecific antibody or a chimeric antibody that bind to said protein.

16. The method of claim 15 wherein said binding reduces/enhances cellular proliferation, differentiation, DNA synthesis or cell cycle diversity.

17. A method for identifying agents that modulate the interaction or the effect of physiological estrogens, stilbene estrogens or triphenylethylene (anti)estrogens with or of the protein of claim 9 comprising the steps of:

a) incubating said protein with a putative inhibitory compound;

b) adding a physiological estrogen, stilbene estrogen or triphenylethylene (anti)estrogen; and

c) determining whether said agent modulates the binding of the protein of claim 9 to said physiological estrogens, stilbene estrogens or triphenylethylene (anti)estrogens.

18. A method to assay for the activation of a protein of claim 9 comprising the step of determining whether said protein is expressed or the step of determining whether the mRNA encoding said protein of claim 9 is expressed.

19. A method to localize a protein of claim 9 *in situ* which method comprises administering to a subject an amount of antibody that binds to said protein.

20. The method of claim 19, wherein the antibody is conjugated to a radioactive isotope.

21. An isolated nucleic acid molecule which is complementary to a nucleic acid molecule of any one of claims 1-3.

22. A method to inhibit expression of the protein of claim 9 in a cell, which comprises providing to said cell the nucleic acid molecule of claim 21 in an amount sufficient to inhibit expression of said protein.

23. A pharmaceutical composition comprising a nucleic acid molecule of claim 20 together with a pharmaceutically acceptable carrier or excipient.

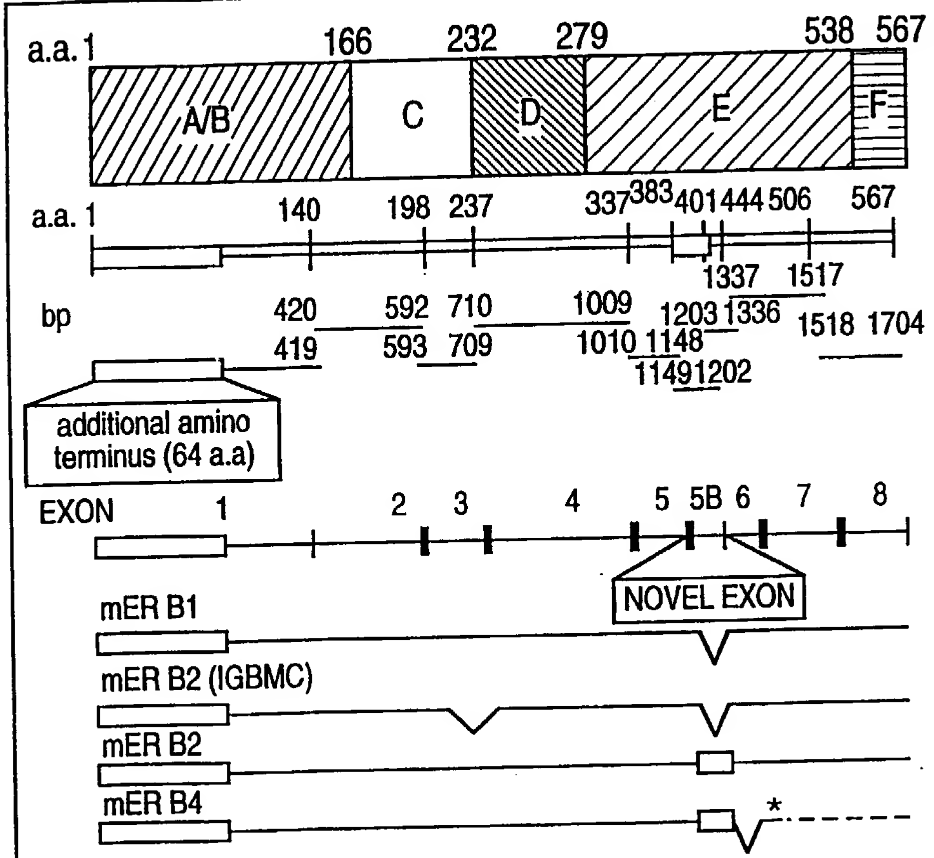
AMENDED CLAIMS

[received by the International Bureau on 08 January 1999 (08.01.99); original claim 23 amended; remaining claims unchanged (1 page)]

22. A method to inhibit expression of the protein of claim 9 in a cell, which comprises providing to said cell the nucleic acid molecule of claim 21 in an amount sufficient to inhibit expression of said protein.

23. A pharmaceutical composition comprising a nucleic acid molecule of claim 21 together with a pharmaceutically acceptable carrier or excipient.

FIG. 1(a)



GENOMIC ORGANIZATION OF THE MURINE BETA ESTROGEN RECEPTOR AND SPLICE VARIANT cDNAs. THE ADDITIONAL 164 BASES OF THE OPEN READING FRAME NOT INCLUDED IN THE ORIGINALLY PUBLISHED CLONE (IN THE A/B TRANSACTIVATION REGION) AND THE NOVEL EXON (IN REGION E, THE LIGAND BINDING DOMAIN) APPEAR AS BOXES. THE STIPPLED LINE AT THE COOH TERMINUS OF mER-b4 IS NOT TRANSLATED BECAUSE OF AN IN FRAME STOP CODON.

FIG. 1(b)

```
    ATGTCCATCTGTGCCTCTTCTCACAAGGATTTTTCTCAGCTGAGACCTACGCAAGACATG
  1 ------+---------+---------+---------+---------+---------+--- 60
    TACAGGTAGACACGGAGAAGAGTGTTCCTAAAAAGAGTCGACTCTGGATGCGTTCTGTAC
    M  S  I  C  A  S  S  H  K  D  F  S  Q  L  R  P  T  Q  D  M  -

    GAGATCAAAAACTCACCATCAAGCCTTACTTCCCCTGCTTCTTATAACTGTAGCCAGTCC
 61 ------+---------+---------+---------+---------+---------+--- 120
    CTCTAGTTTTTGAGTGGTAGTTCGGAATGAAGGGGACGAAGAATATTGACATCGGTCAGG
    E  I  K  N  S  P  S  S  L  T  S  P  A  S  Y  N  C  S  Q  S  -

    ATCCTACCCTTGGAGCATGGTCCCATCTATATCCCTTCCTCCTATGTAGAGAGCCGTCAC
121 ------+---------+---------+---------+---------+---------+--- 180
    TAGGATGGGAACCTCGTACCAGGGTAGATATAGGGAAGGAGGATACATCTCTCGGCAGTG
    I  L  P  L  E  H  G  P  I  Y  I  P  S  S  Y  V  E  S  R  H  -

    GAATACTCAGCCATGACATTCTACAGTCCTGCTGTGATGAACTACAGTGTTCCCAGCAGC
181 ------+---------+---------+---------+---------+---------+--- 240
    CTTATGAGTCGGTACTGTAAGATGTCAGGACGACACTACTTGATGTCACAAGGGTCGTCG
    E  Y  S  A  M  T  F  Y  S  P  A  V  M  N  Y  S  V  P  S  S  -

    ACCGGTAACCTGGAAGGTGGGCCTGTTCGCCAGACTGCAAGCCCAAATGTGCTATGGCCA
241 ------+---------+---------+---------+---------+---------+--- 300
    TGGCCATTGGACCTTCCACCCGGACAAGCGGTCTGACGTTCGGGTTTACACGATACCGGT
    T  G  N  L  E  G  G  P  V  R  Q  T  A  S  P  N  V  L  W  P  -

    ACTTCTGGACACCTCTCTCCTTTAGCCACCCACTGCCAATCATCGCTTCTCTATGCAGAA
301 ------+---------+---------+---------+---------+---------+--- 360
    TGAAGACCTGTGGAGAGAGGAAATCGGTGGGTGACGGTTAGTAGCGAAGAGATACGTCTT
    T  S  G  H  L  S  P  L  A  T  H  C  Q  S  S  L  L  Y  A  E  -

    CCTCAAAAGAGTCCTTGGTGTGAAGCAAGATCACTAGAACACACCTTGCCTGTAAACAGA
361 ------+---------+---------+---------+---------+---------+--- 420
    GGAGTTTTCTCAGGAACCACACTTCGTTCTAGTGATCTTGTGTGGAACGGACATTTGTCT
    P  Q  K  S  P  W  C  E  A  R  S  L  E  H  T  L  P  V  N  R  -
```

FIG. 1(c)

```
    GAGACCCTGAAGAGGAAGCTTGGCGGGAGCGGTTGTGCCAGCCCTGTTACTAGTCCAAGC
421 ------+---------+---------+---------+---------+---------+--- 480
    CTCTGGGACTTCTCCTTCGAACCGCCCTCGCCAACACGGTCGGGACAATGATCAGGTTCG

    E  T  L  K  R  K  L  G  G  S  G  C  A  S  P  V  T  S  P  S  -

    GCCAAGAGGGATGCTCACTTCTGCGCCGTCTGCAGTGATTATGCATCTGGGTATCATTAC
481 ------+---------+---------+---------+---------+---------+--- 540
    CGGTTCTCCCTACGAGTGAAGACGCGGCAGACGTCACTAATACGTAGACCCATAGTAATG

    A  K  R  D  A  H  F  C  A  V  C  S  D  Y  A  S  G  Y  H  Y  -

    GGTGTCTGGTCCTGTGAAGGATGTAAGGCCTTTTTTAAAAGAAGCATTCAAGGACATAAT
541 ------+---------+---------+---------+---------+---------+--- 600
    CCACAGACCAGGACACTTCCTACATTCCGGAAAAAATTTTCTTCGTAAGTTCCTGTATTA

    G  V  W  S  C  E  G  C  K  A  F  F  K  R  S  I  Q  G  H  N  -

    GACTATATCTGTCCAGCCACGAATCAGTGTACCATAGACAAGAACCGGCGTAAAAGCTGC
601 ------+---------+---------+---------+---------+---------+--- 660
    CTGATATAGACAGGTCGGTGCTTAGTCACATGGTATCTGTTCTTGGCCGCATTTTCGACG

    D  Y  I  C  P  A  T  N  Q  C  T  I  D  K  N  R  R  K  S  C  -

    CAGGCCTGCCGACTTCGCAAGTGTTACGAAGTAGGAATGGTCAAGTGTGGATCCAGGAGA
661 ------+---------+---------+---------+---------+---------+--- 720
    GTCCGGACGGCTGAAGCGTTCACAATGCTTCATCCTTACCAGTTCACACCTAGGTCCTCT

    Q  A  C  R  L  R  K  C  Y  E  V  G  M  V  K  C  G  S  R  R  -

    GAAAGGTGTGGGTACCGAATAGTACGAAGACAGAGAAGTGCCAGCGAGCAGGTGCATTGC
721 ------+---------+---------+---------+---------+---------+--- 780
    CTTTCCACACCCATGGCTTATCATGCTTCTGTCTCTTCACGGTCGCTCGTCCACGTAACG

    E  R  C  G  Y  R  I  V  R  R  Q  R  S  A  S  E  Q  V  H  C  -
```

FIG. 1(d)

```
     CTGAACAAAGCCAAGAGAACCAGTGGGCACACACCCCGGGTGAAGGAGCTACTGCTGAAC
 781 ------+---------+---------+---------+---------+---------+--- 840
     GACTTGTTTCGGTTCTCTTGGTCACCCGTGTGTGGGGCCCACTTCCTCGATGACGACTTG
     L  N  K  A  K  R  T  S  G  H  T  P  R  V  K  E  L  L  L  N  -

     TCTCTGAGTCCCGAGCAGCTGGTGCTCACCCTGCTGGAAGCTGAGCCACCCAATGTGCTA
 841 ------+---------+---------+---------+---------+---------+--- 900
     AGAGACTCAGGGCTCGTCGACCACGAGTGGGACGACCTTCGACTCGGTGGGTTACACGAT
     S  L  S  P  E  Q  L  V  L  T  L  L  E  A  E  P  P  N  V  L  -

     GTGAGCCGTCCCAGCATGCCCTTCACCGAGGCCTCCATGATGATGTCCCTCACGAAGCTG
 901 ------+---------+---------+---------+---------+---------+--- 960
     CACTCGGCAGGGTCGTACGGGAAGTGGCTCCGGAGGTACTACTACAGGGAGTGCTTCGAC
     V  S  R  P  S  M  P  F  T  E  A  S  M  M  M  S  L  T  K  L  -

     GCTGACAAGGAACTGGTGCACATGATTGGCTGGGCCAAGAAAATCCCTGGCTTTGTGGAG
 961 ------+---------+---------+---------+---------+---------+--- 1020
     CGACTGTTCCTTGACCACGTGTACTAACCGACCCGGTTCTTTTAGGGACCGAAACACCTC
     A  D  K  E  L  V  H  M  I  G  W  A  K  K  I  P  G  F  V  E  -

     CTCAGCCTGTTGGACCAAGTCCGCCTCTTGGAAAGCTGCTGGATGGAGGTGCTGATGGTG
1021 ------+---------+---------+---------+---------+---------+--- 1080
     GAGTCGGACAACCTGGTTCAGGCGGAGAACCTTTCGACGACCTACCTCCACGACTACCAC
     L  S  L  L  D  Q  V  R  L  L  E  S  C  W  M  E  V  L  M  V  -

     GGGCTGATGTGGCGCTCCATCGACCACCCCGGCAAGCTCATCTTTGCTCCAGACCTCGTT
1081 ------+---------+---------+---------+---------+---------+--- 1140
     CCCGACTACACCGCGAGGTAGCTGGTGGGGCCGTTCGAGTAGAAACGAGGTCTGGAGCAA
     G  L  M  W  R  S  I  D  H  P  G  K  L  I  F  A  P  D  L  V  -
```

FIG. 1(e)

```
     CTGGACAGgtcctcagaagaccctcactggcacgttgcgcagacgaagagtgctgtccca
1141 ------+---------+---------+---------+---------+---------+--- 1200
     GACCTGTCcaggagtcttctgggagtgaccgtgcaacgcgtctgcttctcacgacagggt
     L  D  R  S  S  E  D  P  H  W  H  V  A  Q  T  K  S  A  V  P   -

     aggGATGAGGGGAAGTGCGTGGAAGGGATTCTGGAAATCTTTGACATGCTCCTGGCGACG
1201 ------+---------+---------+---------+---------+---------+--- 1260
     tccCTACTCCCCTTCACGCACCTTCCCTAAGACCTTTAGAAACTGTACGAGGACCGCTGC
     R  D  E  G  K  C  V  E  G  I  L  E  I  F  D  M  L  L  A  T   -

     ACGGCACGGTTCCGTGAGTTAAAACTGCAGCACAAAGAATATCTGTGTGTGAAGGCCATG
1261 ------+---------+---------+---------+---------+---------+--- 1320
     TGCCGTGCCAAGGCACTCAATTTTGACGTCGTGTTTCTTATAGACACACACTTCCGGTAC
     T  A  R  F  R  E  L  K  L  Q  H  K  E  Y  L  C  V  K  A  M   -

     ATTCTCCTCAACTCCAGTATGTACCCCTTGGCTACCGCAAGCCAGGAAGCAGAGAGTAGC
1321 ------+---------+---------+---------+---------+---------+--- 1380
     TAAGAGGAGTTGAGGTCATACATGGGGAACCGATGGCGTTCGGTCCTTCGTCTCTCATCG
     I  L  L  N  S  S  M  Y  P  L  A  T  A  S  Q  E  A  E  S  S   -

     CGGAAGCTGACACACCTATTGAACGCAGTGACAGATGCCCTGGTCTGGGTGATTTCGAAG
1381 ------+---------+---------+---------+---------+---------+--- 1440
     GCCTTCGACTGTGTGGATAACTTGCGTCACTGTCTACGGGACCAGACCCACTAAAGCTTC
     R  K  L  T  H  L  L  N  A  V  T  D  A  L  V  W  V  I  S  K   -

     AGTGGAATCTCTTCCCAGCAGCAGTCAGTCCGTCTGGCCAACCTCCTGATGCTTCTTTCT
1441 ------+---------+---------+---------+---------+---------+--- 1500
     TCACCTTAGAGAAGGGTCGTCGTCAGTCAGGCAGACCGGTTGGAGGACTACGAAGAAAGA
     S  G  I  S  S  Q  Q  Q  S  V  R  L  A  N  L  L  M  L  L  S   -
```

FIG. 1(f)

```
     CATGTCAGGCACATCAGTAACAAGGGCATGGAACATCTGCTCAGCATGAAGTGCAAAAAT
1501 ------+---------+---------+---------+---------+---------+--- 1560
     GTACAGTCCGTGTAGTCATTGTTCCCGTACCTTGTAGACGAGTCGTACTTCACGTTTTTA

     H  V  R  H  I  S  N  K  G  M  E  H  L  L  S  M  K  C  K  N  -

     GTGGTCCCGGTGTACGACCTGCTGCTGGAGATGCTGAATGCTCACACGCTTCGAGGGTAC
1561 ------+---------+---------+---------+---------+---------+--- 1620
     CACCAGGGCCACATGCTGGACGACGACCTCTACGACTTACGAGTGTGCGAAGCTCCCATG

     V  V  P  V  Y  D  L  L  L  E  M  L  N  A  H  T  L  R  G  Y  -

     AAGTCCTCAATCTCGGGGTCTGAGTGCTGCTCGACAGAGGACAGTAAGAGCAAAGAGGGC
1621 ------+---------+---------+---------+---------+---------+--- 1680
     TTCAGGAGTTAGAGCCCCAGACTCACGACGAGCTGTCTCCTGTCATTCTCGTTTCTCCCG

     K  S  S  I  S  G  S  E  C  C  S  T  E  D  S  K  S  K  E  G  -

     TCCCAGAACCTCCAGTCACAGTGA
1681 ------+---------+------- 1704
     AGGGTCTTGGAGGTCAGTGTCACT
```

FIG. 2(a)

```
  1 MSICASSHKD FSQLRPTQDM EIKNSPSSLT SPASYNCSQS ILPLEHGPIY
 51 IPSSYVESRH EYSAMTFYSP AVMNYSVPSS TGNLEGGPVR QTASPNVLWP
101 TSGHLSPLAT HCQSSLLYAE PQKSPWCEAR SLEHTLPVNR ETLKRKLGGS
151 GCASPVTSPS AKRDAHFCAV CSDYASGYHY GVWSCEGCKA FFKRSIQGHN
201 DYICPATNQC TIDKNRRKSC QACRLRKCYE VGMVKCGSRR ERCGYRIVRR
251 QRSASEQVHC LNKAKRTSGH TPRVKELLLN SLSPEQLVLT LLEAEPPNVI
301 VSRPSMPFTE ASMMMSLTKL ADKELVHMIG WAKKIPGFVE LSLLDQVRLL
351 ESCWMEVLMV GLMWRSIDHP GKLIFAPDLV LDRDEGKCVE GILEIFDMLL
401 ATTARFRELK LQHKEYLCVK AMILLNSSMY PLATASQEAE SSRKLTHLLN
451 AVTDALVWVI SKSGISSQQQ SVRLANLLML LSHVRHISNK GMEHLLSMKC
501 KNVVPVYDLL LEMLNAHTLR GYKSSISGSE CCSTEDSKSK EGSQNLQSQ*
```

FIG. 2(b)

1 MSICASSHKD FSQLRPTQDM EIKNSPSSLT SPASYNCSQS ILPLEHGPIY

51 IPSSYVESRH EYSAMTFYSP AVMNYSVPSS TGNLEGGPVR QTASPNVLWP

101 TSGHLSPLAT HCQSSLLYAE PQKSPWCEAR SLEHTLPVNR ETLKRKLGGS

151 GCASPVTSPS AKRDAHFCAV CSDYASGYHY GVWSCEGCKA FFKRSIQGSR

201 RERCGYRIVR RQRSASEQVH CLNKAKRTSG HTPRVKELLL NSLSPEQLVL

251 TLLEAEPPNV LVSRPSMPFT EASMMMSLTK LADKELVHMI GWAKKIPGFV

301 ELSLLDQVRL LESCWMEVLM VGLMWRSIDH PGKLIFAPDL VLDRDEGKCV

351 EGILEIFDML LATTARFREL KLQHKEYLCV KAMILLNSSM YPLATASQEA

401 ESSRKLTHLL NAVTDALVWV ISKSGISSQQ QSVRLANLLM LLSHVRHISN

451 KGMEHLLSMK CKNVVPVYDL LLEMLNAHTL RGYKSSISGS ECCSTEDSKS

501 KEGSQNLQSQ

FIG. 2(c)

1 **MSICTSSHKE FSQLRPSEDM EIKNSPSSLS SPASYNCSQS ILPLEHGPIY**

51 **IPSSYVDNRH EYSA**MTFYSP AVMNYSVPGS TSNLDGGPVR LSTSPNVLWP

101 TSGHLSPLAT HCQSSLLYAE PQKSPWCEAR SLEHTLPVNR ETLKRKLSGS

151 SCASPVTSPN AKRDAHFCPV CSDYASGYHY GVWSCEGCKA FFKRSIQGHN

201 DYICPATNQC TIDKNRRKSC QACRLRKCYE VGMVKCGSRR ERCGYRIVRR

251 QRSSSEQVHC LSKAKRNGGH APRVKELLLS TLSPEQLVLT LLEAEPPNVL

301 VSRPSMPFTE ASMMMSLTKL ADKELVHMIG WAKKIPGFVE LSLLDQVRLL

351 ESCWMEVLMV GLMWRSIDHP GKLIFAPDLV LDR**SSEDPHW HVAQMKSAAP**

401 RYVPLGFCKP GGRK*PEADT PTERGDRCPG LGDCEEWYLL PAAVSPTGQP

451 PDASFSRQAH Q*QGHGTSAQ HEVQKCGPGV *PAAGDAECS HASRVQVLNL

501 GV*VQLNRGQ *EQRELPEPT VSVMARPEAD RLQRWSKVEH VP*HLGVPLR

551 AALVTHPLPT LHFPGVRVVV WRCSSYQDVP PNAKF*LV*P *RLSVYLLSV

601 SLPTWKHLKG SGTKGQSLIW KDCP*SGKGI WHVTQL*EMD CRTVWP*NQP

651 LDGVF*TT*L *D*KPH*QSA HFAFLPHGSV RTH*CHGLFY QRPER*CKLR

701 CTLFLLPDPW VTSLEPAYLV CLNVVIVMG* DLNLFVILAS LKLCHLSLSP

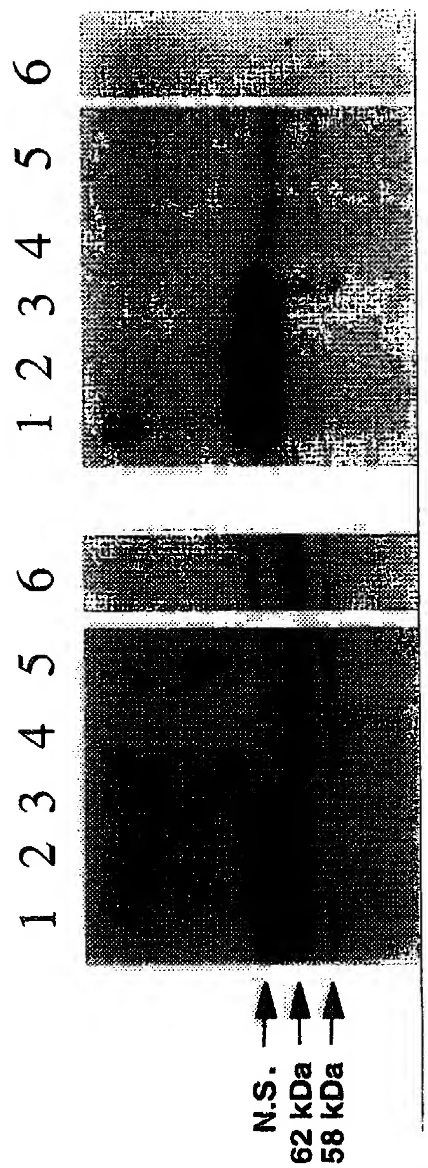


FIG. 3(a)

FIG. 3(b)

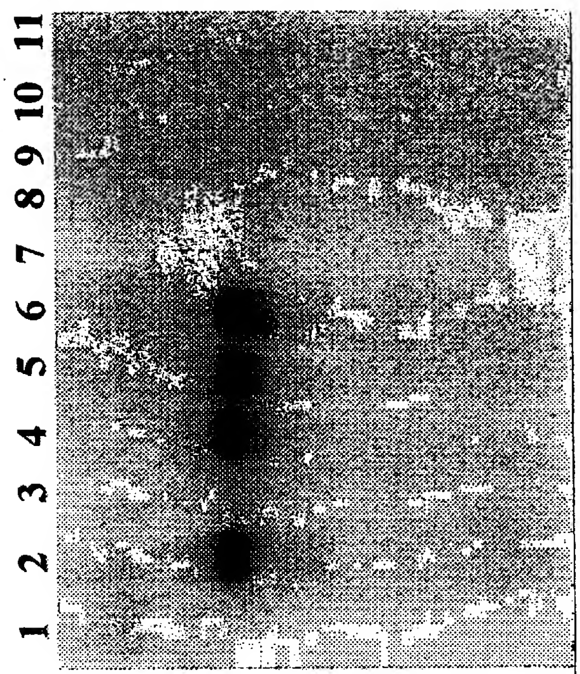


FIG. 4(b)

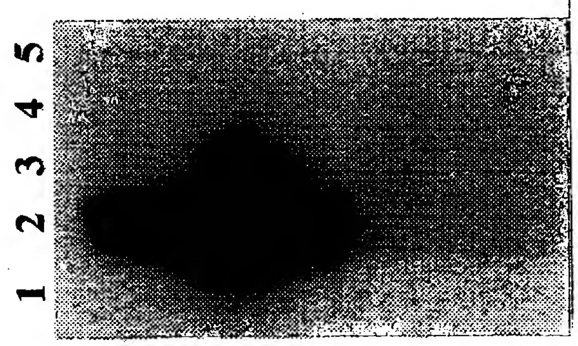


FIG. 4(a)

FIG. 5(a)

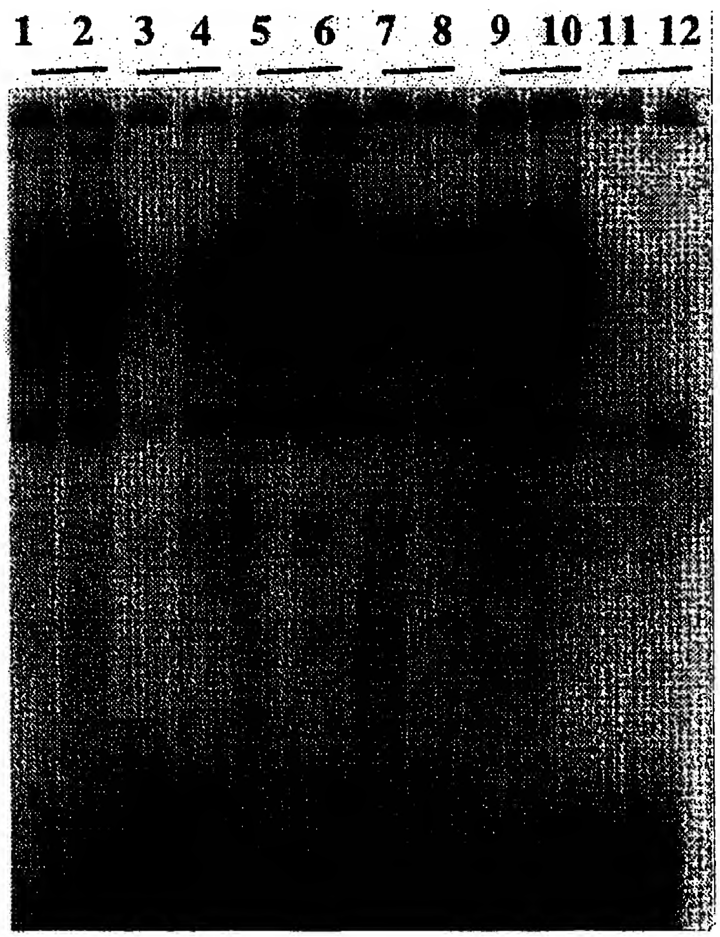


FIG. 5(b)

FIG. 6

```
  1  ....MSICASSHKDFSQLRPTQDMEIKNSP.......SSLTSPASYNCSQ  39
  1  MTMTLHTKASGMALLHQIQ.GNELEPLNRPQLKMPMERALGEVYVDNSKP  49

 40  SILPLEHGPIYIPSSYVESRHEYSAMTFYSPAVMNYSVPSSTGNLEGGPV  89
 50  TVFNYPEGAAYEFNAAAAAAAASA.PVYGQSGIAYGPGSEAAAFSANSL  98

 90  .......RQTASPNVLWPTSGHLSP.LATHCQSSLLYAEPQKSPWC....  127
 99  GAFPQLNSVSPSPLMLLHPPPQLSPFLHPHGQQVPYYLENEPSAYAVRDT  148

128  EARSLEHTLPVN.RETLKRKLGGSGCASPVTSPSAKRDAHFCAVCSDYAS  176
149  GPPAFYRSNSDNRRQNGRERLSSSNEKGNMIMESAK.ETRYCAVCNDYAS  197

177  GYHYGVWSCEGCKAFFKRSIQGHNDYICPATNQCTIDKNRRKSCQACRLR  226
198  GYHYGVWSCEGCKAFFKRSIQGHNDYMCPATNQCTIDKNRRKSCQACRLR  247

227  KCYEVGMVKCGSRRERCGYRIV..RRQRSASEQVHCLNKAKRTSGHTPRV  274
248  KCYEVGMMKGGIRKDRRGGRMLKHKRQRDDLEGRNEMGASGDMRAANLWP  297

275  KELLLN.........SLSPEQLVLTLLEAEPPNVLVS.RPSMPFTEASMM  314
298  SPLVIKHTKKNSPALSLTADQMVSALLDAEPPMIYSEYDPSRPFSEASMM  347

315  MSLTKLADKELVHMIGWAKKIPGFVELSLLDQVRLLESCWMEVLMVGLMW  364
348  GLLTNLADRELVHMINWAKRVPGFGDLNLHDQVHLLECAWLEILMIGLVW  397

365  RSIDHPGKLIFAPDLVLDRDEGKCVEGILEIFDMLLATTARFRELKLQHK  414
398  RSMEHPGKLLFAPNLLLDRNQGKCVEGMVEIFDMLLATSSRFRMMNLQGE  447

415  EYLCVKAMILLNSSMYP.LATASQEAESSRKLTHLLNAVTDALVWVISKS  463
448  EFVCLKSIILLNSGVYTFLSSTLKSLEEKDHIHRVLDKITDTLIHLMAKA  497

464  GISSQQQSVRLANLLMLLSHVRHISNKGMEHLLSMKCKNVVPVYDLLLEM  513
498  GLTLQQQHRRLAQLLLILSHIRHMSNKGMEHLYNMKCKNVVPLYDLLLEM  547

514  LNAHTLRGYKS..SISGSECCSTE.DSKSKEGSQNLQSQ...........  549
548  LDAHRLHAPASRMGVPPEEPSQTQLATTSSTSAHSLQTYYIPPEAEGFPN  597
```

FIG. 7(a)

```
151 ATCCCTTCCTCCTATGTAGAGAGCCGTCACGAATACTCAGCCATGACATT 200
                                 |   | |||||||||
  1 ...............................TCGAGGTCGACATGACATT 19

201 CTACAGTCCTGCTGTGATGAACTACAGTGTTCCCAGCAGCACCGGTAACC 250
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 20 CTACAGTCCTGCTGTGATGAACTACAGTGTTCCCAGCAGCACCGGTAACC 69

251 TGGAAGGTGGGCCTGTTCGCCAGACTGCAAGCCCAAATGTGCTATGGCCA 300
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 70 TGGAAGGTGGGCCTGTTCGCCAGACTGCAAGCCCAAATGTGCTATGGCCA 119

301 ACTTCTGGACACCTCTCTCCTTTAGCCACCCACTGCCAATCATCGCTTCT 350
    ||||||||||||||||||||||||||||||||||||||||||||||||||
120 ACTTCTGGACACCTCTCTCCTTTAGCCACCCACTGCCAATCATCGCTTCT 169

351 CTATGCAGAACCTCAAAAGAGTCCTTGGTGTGAAGCAAGATCACTAGAAC 400
    ||||||||||||||||||||||||||||||||||||||||||||||||||
170 CTATGCAGAACCTCAAAAGAGTCCTTGGTGTGAAGCAAGATCACTAGAAC 219

401 ACACCTTGCCTGTAAACAGAGAGACCCTGAAGAGGAAGCTTGGCGGGAGC 450
    ||||||||||||||||||||||||||||||||||||||||||||||||||
220 ACACCTTGCCTGTAAACAGAGAGACCCTGAAGAGGAAGCTTGGCGGGAGC 269

451 GGTTGTGCCAGCCCTGTTACTAGTCCAAGCGCCAAGAGGGATGCTCACTT 500
    ||||||||||||||||||||||||||||||||||||||||||||||||||
270 GGTTGTGCCAGCCCTGTTACTAGTCCAAGCGCCAAGAGGGATGCTCACTT 319

501 CTGCGCCGTCTGCAGTGATTATGCATCTGGGTATCATTACGGTGTCTGGT 550
    ||||||||||||||||||||||||||||||||||||||||||||||||||
320 CTGCGCCGTCTGCAGTGATTATGCATCTGGGTATCATTACGGTGTCTGGT 369

551 CCTGTGAAGGATGTAAGGCCTTTTTAAAAGAAGCATTCAAGGACATAAT 600
    ||||||||||||||||||||||||||||||||||||||||||||||||||
370 CCTGTGAAGGATGTAAGGCCTTTTTAAAAGAAGCATTCAAGGACATAAT 419

601 GACTATATCTGTCCAGCCACGAATCAGTGTACCATAGACAAGAACCGGCG 650
    ||||||||||||||||||||||||||||||||||||||||||||||||||
420 GACTATATCTGTCCAGCCACGAATCAGTGTACCATAGACAAGAACCGGCG 469

651 TAAAAGCTGCCAGGCCTGCCGACTTCGCAAGTGTTACGAAGTAGGAATGG 700
    ||||||||||||||||||||||||||||||||||||||||||||||||||
470 TAAAAGCTGCCAGGCCTGCCGACTTCGCAAGTGTTACGAAGTAGGAATGG 519

701 TCAAGTGTGGATCCAGGAGAGAAAGGTGTGGGTACCGAATAGTACGAAGA 750
    ||||||||||||||||||||||||||||||||||||||||||||||||||
```

FIG. 7(b)

```
 520 TCAAGTGTGGATCCAGGAGAGAAAGGTGTGGGTACCGAATAGTACGAAGA 569
              .         .         .         .         .
 751 CAGAGAAGTGCCAGCGAGCAGGTGCATTGCCTGAACAAAGCCAAGAGAAC 800
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 570 CAGAGAAGTGCCAGCGAGCAGGTGCATTGCCTGAACAAAGCCAAGAGAAC 619
              .         .         .         .         .
 801 CAGTGGGCACACACCCCGGGTGAAGGAGCTACTGCTGAACTCTCTGAGTC 850
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 620 CAGTGGGCACACACCCCGGGTGAAGGAGCTACTGCTGAACTCTCTGAGTC 669
              .         .         .         .         .
 851 CCGAGCAGCTGGTGCTCACCCTGCTGGAAGCTGAGCCACCCAATGTGCTA 900
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 670 CCGAGCAGCTGGTGCTCACCCTGCTGGAAGCTGAGCCACCCAATGTGCTA 719
              .         .         .         .         .
 901 GTGAGCCGTCCCAGCATGCCCTTCACCGAGGCCTCCATGATGATGTCCCT 950
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 720 GTGAGCCGTCCCAGCATGCCCTTCACCGAGGCCTCCATGATGATGTCCCT 769
              .         .         .         .         .
 951 CACGAAGCTGGCTGACAAGGAACTGGTGCACATGATTGGCTGGGCCAAGA 1000
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 770 CACGAAGCTGGCTGACAAGGAACTGGTGCACATGATTGGCTGGGCCAAGA 819
              .         .         .         .         .
1001 AAATCCCTGGCTTTGTGGAGCTCAGCCTGTTGGACCAAGTCCGCCTCTTG 1050
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 820 AAATCCCTGGCTTTGTGGAGCTCAGCCTGTTGGACCAAGTCCGCCTCTTG 869
              .         .         .         .         .
1051 GAAAGCTGCTGGATGGAGGTGCTGATGGTGGGGCTGATGTGGCGCTCCAT 1100
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 870 GAAAGCTGCTGGATGGAGGTGCTGATGGTGGGGCTGATGTGGCGCTCCAT 919
              .         .         .         .
1101 CGACCACCCCGGCAAGCTCATCTTTGCTCCAGACCTCGTTCTGGACAGGT 1150
     ||||||||||||||||||||||||||||||||||||||||||||||
 920 CGACCACCCCGGCAAGCTCATCTTTGCTCCAGACCTCGTTCTGGAC.... 965
                          .
                          .
                          .
```

FIG. 7(c)

```
1201 AGGGATGAGGGGAAGTGCGTGGAAGGGATTCTGGAAATCTTTGACATGCT 1250
     ||||||||||||||||||||||||||||||||||||||||||| ||||||
 966 AGGGATGAGGGGAAGTGCGTGGAAGGGATTCTGGAAATCTTTGGCATGCT 1015

1251 CCTGGCGACGACGGCACGGTTCCGTGAGTTAAAACTGCAGCACAAAGAAT 1300
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1016 CCTGGCGACGACGGCACGGTTCCGTGAGTTAAAACTGCAGCACAAAGAAT 1065

1301 ATCTGTGTGTGAAGGCCATGATTCTCCTCAACTCCAGTATGTACCCCTTG 1350
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1066 ATCTGTGTGTGAAGGCCATGATTCTCCTCAACTCCAGTATGTACCCCTTG 1115

1351 GCTACCGCAAGCCAGGAAGCAGAGAGTAGCCGGAAGCTGACACACCTATT 1400
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1116 GCTACCGCAAGCCAGGAAGCAGAGAGTAGCCGGAAGCTGACACACCTATT 1165

1401 GAACGCAGTGACAGATGCCCTGGTCTGGGTGATTTCGAAGAGTGGAATCT 1450
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1166 GAACGCAGTGACAGATGCCCTGGTCTGGGTGATTTCGAAGAGTGGAATCT 1215

1451 CTTCCCAGCAGCAGTCAGTCCGTCTGGCCAACCTCCTGATGCTTCTTTCT 1500
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1216 CTTCCCAGCAGCAGTCAGTCCGTCTGGCCAACCTCCTGATGCTTCTTTCT 1265

1501 CATGTCAGGCACATCAGTAACAAGGGCATGGAACATCTGCTCAGCATGAA 1550
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1266 CATGTCAGGCACATCAGTAACAAGGGCATGGAACATCTGCTCAGCATGAA 1315

1551 GTGCAAAAATGTGGTCCCGGTGTACGACCTGCTGCTGGAGATGCTGAATG 1600
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1316 GTGCAAAAATGTGGTCCCGGTGTACGACCTGCTGCTGGAGATGCTGAATG 1365

1601 CTCACACGCTTCGAGGGTACAAGTCCTCAATCTCGGGGTCTGAGTGCTGC 1650
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1366 CTCACACGCTTCGAGGGTACAAGTCCTCAATCTCGGGGTCTGAGTGCTGC 1415

1651 TCGACAGAGGACAGTAAGAGCAAAGAGGGCTCCCAGAACCTCCAGTCACA 1700
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1416 TCGACAGAGGACAGTAAGAGCAAAGAGGGCTCCCAGAACCTCCAGTCACA 1465

1701 GTGA.............................................. 1704
     ||||
1466 GTGACGGCCAGGCTGGAGGCGGATGATTACAATGATGGTCAGAAGTGGGA 1515
```

FIG. 8

B3 IS DOMINANT NEGATIVE, BUT IT MAY BE CONDITIONAL
100 nM DRUG COS-7

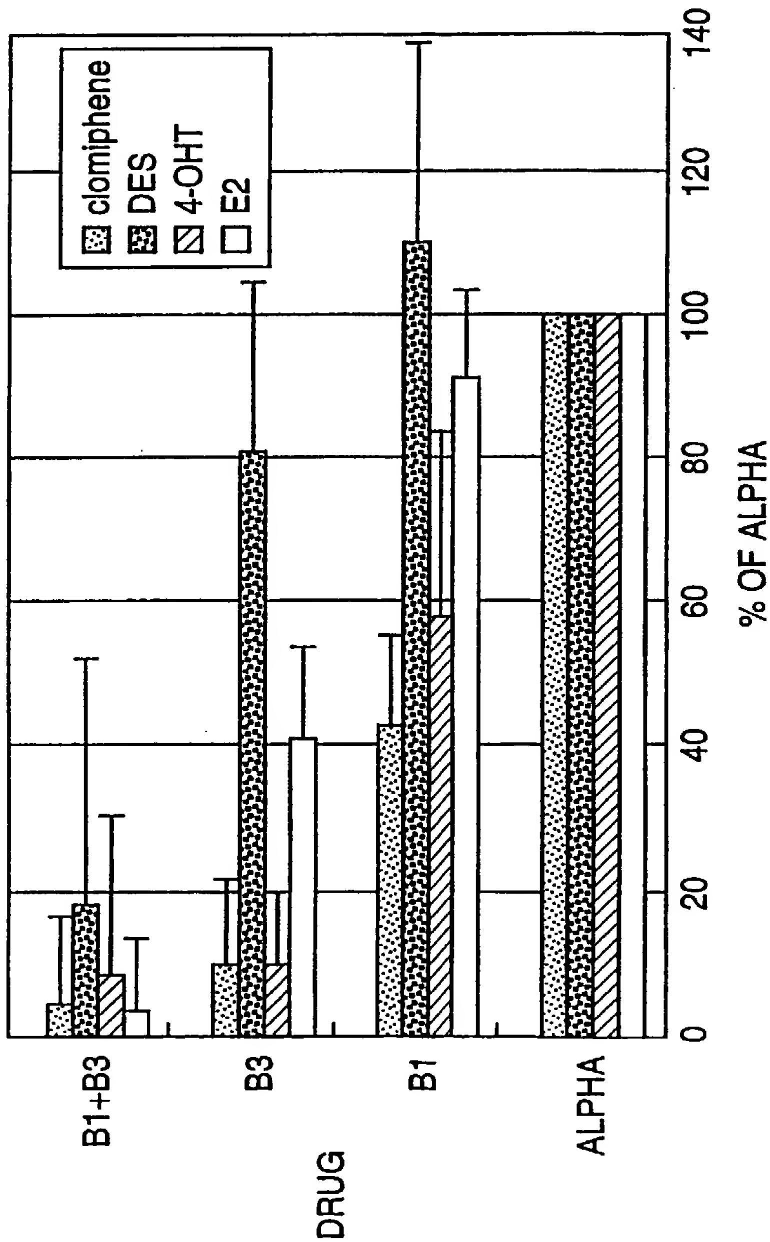


FIG. 9(b)

mER-B1

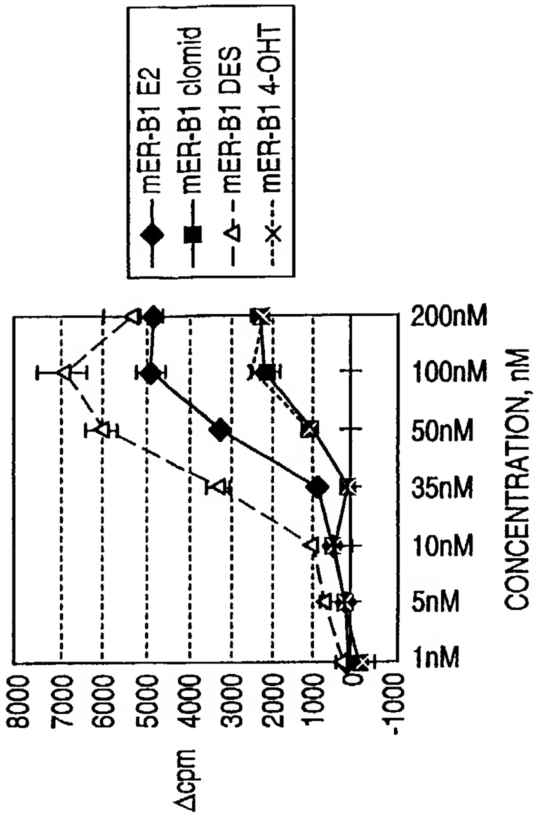


FIG. 9(a)

ER ALPHA

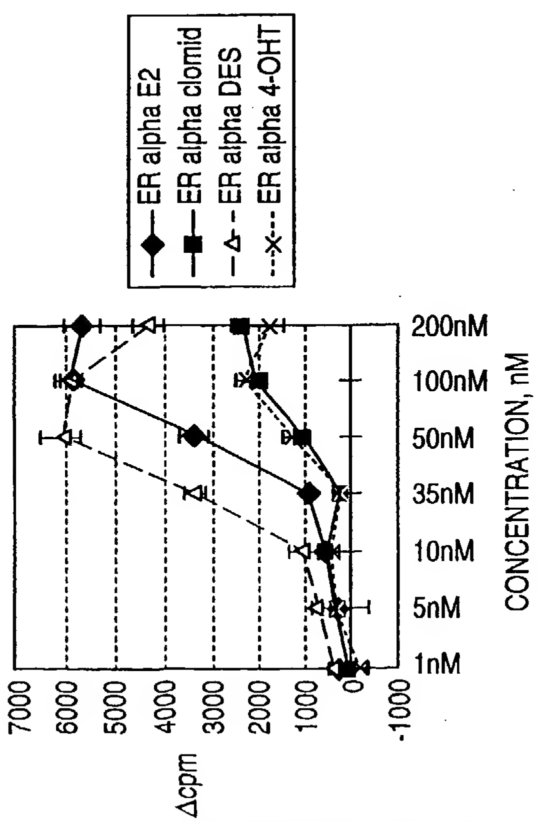


FIG. 9(c)

mER-B3

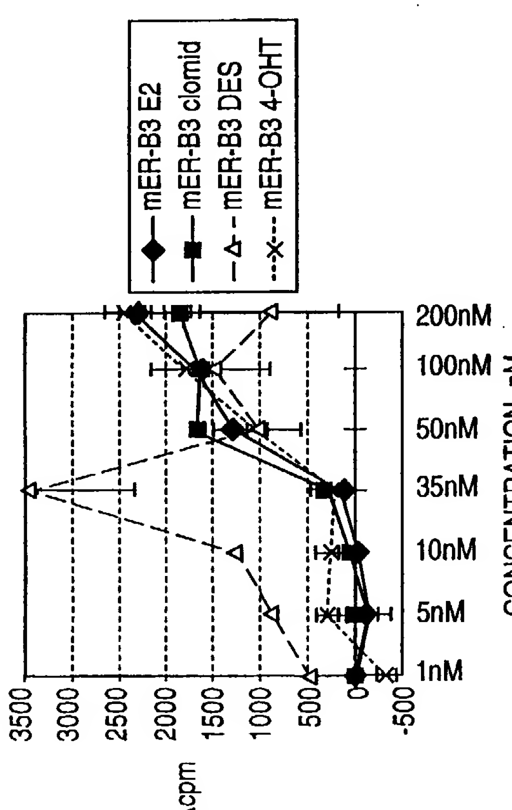


FIG. 9(d)

mER B1+B3

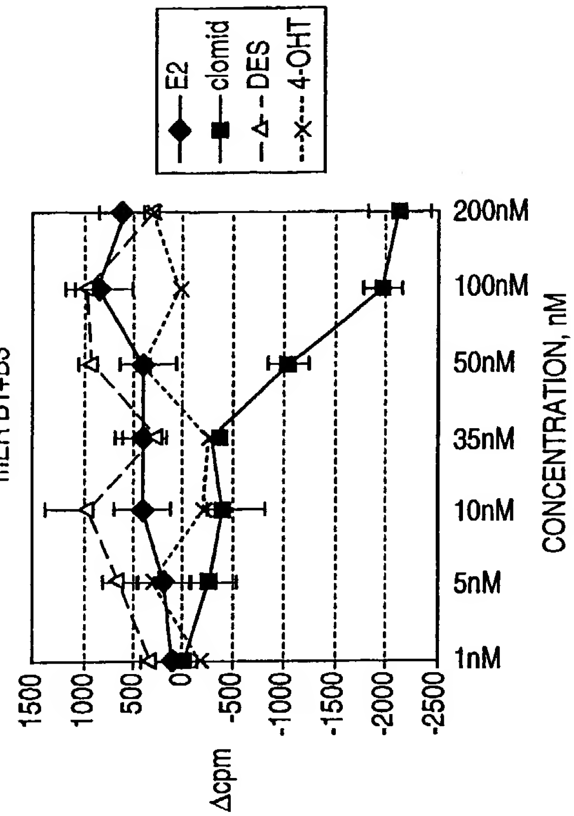


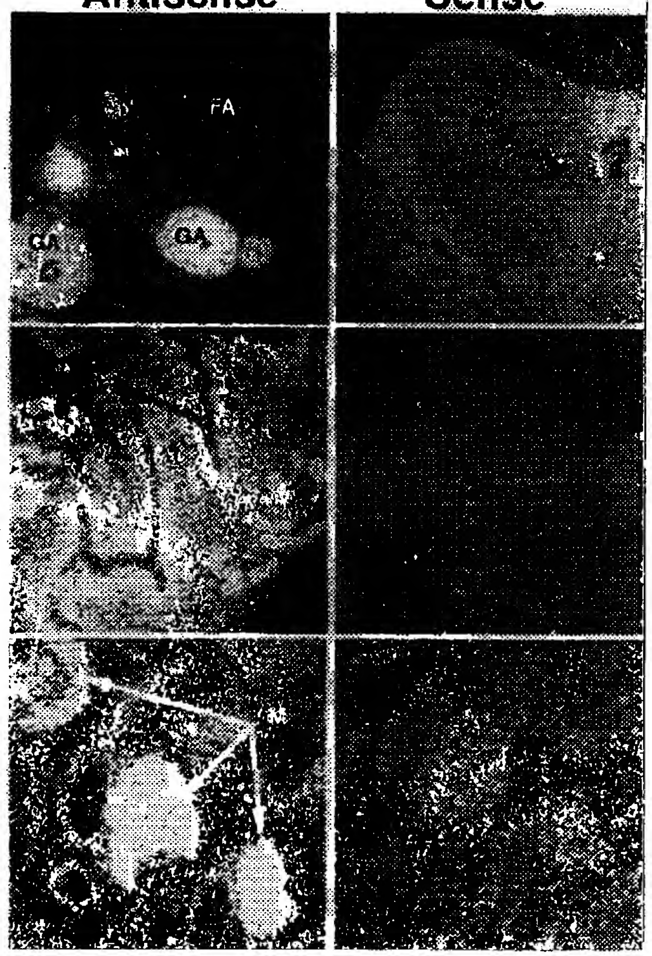
FIG. 10

Antisense Sense

Beta Ovary

Alpha Uterus

Beta Spine



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15540

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 14/435, 14/47, 14/705; C12N 1/21, 5/10 , 15/10, 15/11
US CL :435/69.1, 252.3, 325; 530/350; 536/23.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 252.3, 325; 530/350; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN, BIOSCIENCE, MEDLINE

search terms: estrogen?(5a)receptor?, beta

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOSSELMAN et al. ERbeta: identifiaction and characterization of a novel human estrogen receptor. FEBS letters. 19 August 1996, Vol. 392, No. 1, pages 49-53, especially pages 49, 50, and 52.	1-9, 21
X	TREMBLAY et al. Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta. Molecular Endocrinology. March 1997, Vol. 11, No. 3, pages 353-365, especially abstract.	1-9, 21
X	KUIPER et al. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc. Natl. Acad. Sci. USA. June 1996, Vol. 93, 5925-5930, especially pages 5925, 5926, 5928, and 5928.	1-9, 21

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 OCTOBER 1998	12 NOV 1998

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	MICHAEL D. PAK Della Collins for
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15540

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 9 (IN PART)
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Please See Extra Sheet.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

(See attached PCT telephone memorandum)

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-8,9 in part and 21

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15540

BOX I. OBSERVATIONS WHERE CLAIMS WERE FOUND UNSEARCHABLE
2. Where no meaningful search could be carried out, specifically:

The claim is unsearchable to the extent that they require reference to the specified sequences from the sequence listing. Because Applicant has not furnished a machine-readable copy of the sequence listing as required by PCT Rule 5.2, no meaningful search of the sequences per se can be carried out by this Authority. However, the subject matter of the claims has been searched to the extent possible with reference to the balance of the description.